ACTA PHARMACOLOGICA ET TOXICOLOGICA

IUSSU SOCIETATIS PHARMACOLOGICAE SCANDINAVICAE EDITA

VOL. 28 — 1970

MUNKSGAARD
COPENHAGEN

VOL. 28 INDEX

FASC. 1 (NOVEMBER 1969)

SCHELL KRÜGER, J. Central Effects of Anticholinergic Drugs Measured by the Apomorphine Gnawing Test in Mice ... 1–16

HERMANSEN, KELD: Antifibrillatory Effect of Some β-Adrenergic Receptor Blocking Agents Determined by a New Test Procedure in Mice ... 17–27

BAKKE, OLAV M. O-Methylation of Simple Phenols in the rat ... 28–38

ANDÉN, NILS-ERIK, SHERREL G. BUTCHER & KJELL FUXE: Protection of the Neostriatal Dopamine Stores against Reserpine by Local Treatment with Metatyramine ... 39–48

PERSSON, HENRY & BIRGITTA JOHNSON: Adrenergic Receptors in the Guinea Pig Trachea and Lung ... 49–56

HERMANSEN, KELD: Evidence for Adrenergic Mediation of Ouabain Induced Arrhythmias in the Guinea Pig ... 57–65

SVEDMYR, NILS & LENNART HARTHON: Comparison between the Absorption of Nicotinic Acid and Pentaerythritol Tetranicotinate (Perycit®) from Ordinary and Enterocoated Tablets ... 66–74

STÅLHANDSKE, TORBJÖRN & PRIYAVRAT SLANINA: Effect of Nicotine Treatment on the Metabolism of Nicotine in the Mouse Liver in Vitro ... 75–80

FASC. 2 (FEBRUARY 1970)

FREDERICSON OVERØ, K., A. JØRGENSEN & V. HANSEN: Metabolism, Distribution and Excretion of the Thiophthalane Lu 5-003, Bicyclic Thymoleptic ... 81–96

SECHER-HANSEN, E. Studies on the Subcutaneous Absorption in Mice VII. Absorption of ^3H_2O and ^{14}C-sucrose from Non-buffered Solutions at Different pH Values ... 97–101

SECHER HANSEN, E. Studies on the Subcutaneous Absorption in Mice VIII. Influence of Connective Tissue Ground Substance on the Absorption of Subcutaneously Injected Depots of Sodium and Chloride ... 102–107

WINTHER, FINN Ø. Experimentally Induced Cough in Man by Citric Acid Aerosol. An Evaluation of a Method ... 108–112

VENDSBORG, P. B. & P. SCHAMBYE: The Influence of 2,4-Dinitrophenol on Metabolic Changes Caused by Ethanol in the Perfused Rat Liver ... 113–123

BRISEID, KJELL, OLAV KJELL DYRUD & SVEIN ÖIE: Determination of Factors of the Kinin System in Rat Plasma ... 124–137

BRISEID, KJELL, OLAV KJELL DYRUD & FINN CATO ARNTZEN: Substrates for Kinin-Releasing Enzymes in Rat Plasma ... 138–144

SELYE, HANS: Prevention of Mephenesin Intoxication by Catatoxic Steroids ... 145–148

BOYD, E. M. & F. M. LIU: Maximal Tolerated Daily Doses of Dicophane ... 149–160

FASC. 3 (MAY 1970)

Elwin, Carl Eric: Emetic and Gastric Secretory Stimulating Action by Esters of some Amino Acids .. 161–176
Chandra, Satya V & S. P Srivastava *Experimental Production of Early Brain Lesions in Rats by Parenteral Administration of Manganese Chloride* .. 177–183
Skinhøj Annette: The Blood Pressure Response Following Angiotensin in Nephrectomized, Urethanized Rats .. 184–191
Holm, Jane: The Uptake of Decamethonium and Hexamethonium by Slices of Mouse Kidney .. 192 202
Wilhelm, Benoit J & E. Jacobsen: The Protective Action of Different Barbituric Acid Derivatives against Anoxia in Mice .. 203–208
Franksson, Gunhild & Erik Änggård: The Plasma Protein Binding of Amphetamine, Catecholamines and Related Compounds .. 209–214
Broen Christensen, C: In Vitro Studies on the Uptake of 14-Labelled Decamethonium and Hexamethonium in Mouse Liver .. 215–224
Bristed, Gudrun: An Assay Method for Trasylol® Based on the In Vitro Inhibition of Human Plasma Kallikrein .. 225–232
Ståhlhandske, Torbjörn & Přemysl Slanina. Lethal Brain Concentrations of Nicotine in Mice of Different Ages .. 232 240
Petersen, P V N Larsen, T Ammitzbøll, L Møller Nielsen, M. Nymark, V Pedersen & K. F Franck. Further Pharmacological Studies of Bicylic Thymoleptics .. 241 248

FASC. 4 (JULY 1970)

Åberg, G & K. S. Larsson: Pharmacological Properties of Some Antirheumatic Salicylates .. 249–257
Langslet Asbjørn: ECG-Changes Induced by Phenothiazine Drugs in the Anaesthetized Rat .. 258–264
Olesen O Vendelin & O. Nygaard Jensen: The Influence of Folic Acid on Phenytoin (DPH) Metabolism and the 24-Hours Fluctuation in Urinary Output of 5-(p-hydroxyphenyl)-5 phenyl-hydantoin (HPPH) .. 265–269
Jensen-Holm, Jens & Per Juul. The Effects of Guanethidine, Pre- and Post ganglionic Nerve Division on the Rat Superior Cervical Ganglion. Cholinesterase and Protein .. 270–282
Jensen-Holm, Jens & Per Juul. The Effects of Guanethidine, Pre and Post ganglionic Nerve Division on the Rat Superior Cervical Ganglion. Cholinesterases and Catecholamines (Histochemistry), and Histology .. 283–298
Airaksinen, Mauno M Per H. Rosenberg & Tapani Tammisto. A Possible *Mechanism of Toxicity of Trifluoroethanol and Other Halothane Metabolites* 299–304
Albanus, Lennart: Central and Peripheral Effects of Anticholinergic Compounds .. 305–326

FASC. 5 (SEPTEMBER 1970)

Rosenberg, Per H., Mauno M. Airaksinen & Tapani Tammisto: Inhibition of Energy Production by Halothane Metabolites .. 327–333

FELBY S., Concentrations of Meprobamate in the Blood and Liver Following Fatal Meprobamate Poisoning ... 334–337
SRINIVASAN, S., U. SRINIVASAN & J. H. BALWANI: Effect of Thyroxine, Reserpine and Serotonin on Allyl Alcohol Induced Hepatotoxicity in Rats ... 338–345
TOMPSETT, S. L., Paraquat Poisoning ... 346–358
BARTONÍČEK, V. J. & A. BRUN: Subacute and Chronic Trichloroethylene Poisoning: A Neuropathological Study in Rabbits ... 359–369
HOLM, JAN: The Effect of d-Tubocurarine on the Kinetics of Decamethonium Uptake by Mouse Kidney Slices ... 370–377
PERSSON, TORGNY: Drug Induced Changes in ³H-Catecholamine Accumulation after ³H-Tyrosine ... 378–390
RYRFELDT, ÅKE: The Distribution, Elimination, and Biotransformation of ¹⁴C-Cloforex in the Mouse and Rat ... 391–405
WALDECK, BERTIL, Failure to Demonstrate Monoamine Oxidase Inhibition by Glyceryl Trinitrat in Vivo ... 406–412

FASC. 6 (NOVEMBER 1970)

NIELSEN, GYDA H., Percutaneous Absorption of Boric Acid from Boron-Containing Preparations in Rats ... 413–424
VAN EEKEN, C. J., R. D. R. BIRTWHISTLE & D. MULDER, Toxicity Studies with Fluindarol, 2-[p-(trifluoromethyl)phenyl]-1,3-indandione, an Agent with Anticoagulant Properties (BS 7616D) ... 425–434
MAGNUSSON, GÖRAN, HANS CORNBOCK & ERKIL HANSSON: Renal Lesions Induced by α-Methyltyrosine Methylester and α-Methyltyrosine ... 435–444
AKERMAN, B. & S. ROSS: Stereospecificity of the Enzymatic Biotransformation of the Enantiomers of Prilocaine (Citanest®) ... 445–453
VESTERÅDO, ANNE MARIE: Effects of Tetraethylthiuram Disulphide (Disulfiram), Diethyldithiocarbamate and Ethanol on Factors of the Kinin System in Human Blood ... 454–465
PERSSON, TORGNY & BERTIL WALDECK: A Source of Error in Catecholamine Turnover Studies with Labelled DOPA. Slow Disappearance of the Precursor 466–476
FREY H.-H. & R. SCHULZ: Time Course of the Demethylation of Trimethadione 477–483
RAVN-JONSEN, A., The Urinary Excretion of Enfbomalum (NFN) in Man after Administration of Anaesthetic Doses ... 484–492
WAHLSTRÖM, GÖRAN, HORST BÜCH & WALTER BUZELLO: Unequal Anaesthetic Potency Despite Equal Brain Concentration of Hexobarbital Antipodes 493–498
PANDYA, K. P., G. B. SINGH & N. C. JOSHI: Effect of Benzanthrone on the Body Level of Ascorbic Acid in Guinea Pigs ... 499–506

SUPPLEMENTUM 1

XXIV Scandinavian Pharmacological Meeting, Oslo 1970. Abstracts.

INDEX

Absorption, anticholinergics, Suppl. 1 14
– oral, nicotinic acid and pentaerythritol tetranicotinate, 66
– percutaneous, boric acid, 413
–, subcutaneous and connective tissue ground substance, 102
– – at different pH, 97
Acetylcholine biosynthesis, Suppl. 1, 80
Acetylcholinesterase, activation of Tris, Suppl 1, 55
Acetylcholine turnover drug effects, Suppl. 1, 81
Adipose tissue metabolism, theophylline effect, Suppl 1 46
Adrenaline response and cyclic 3'5' AMP Suppl. 1, 93
Adrenergic blockers, antifibrillatory effect, 17
Adrenergic mediation of oubain induced arrhythmias, 57
– receptor blockers and stimulants, 49
– response inhibition, Suppl. 1 60
Ahrén, Kurt, Suppl. 1, 53
Ahtee, Liisa, Suppl. 1, 29
Airaksinen, Mauno M., 299 327
Albanus, Lennart, 305
Alcohol, meprobamate and barbiturate interaction, 334
Alexanderson, Baltzar Suppl. 1, 11
Allyl alcohol hepatotoxicity 338
Amino acid esters, gastric secretion, 161
Ammitzbøll, T 241
3'5'-AMP cyclic, Suppl. 1 45
– effects on heart, Suppl. 1, 53
Amphetamine abuse, pharmacokinetics, Suppl. 1 92
– metabolism and catecholamines, Suppl. 1, 62
– – in brain, Suppl. 1, 62
– response and monoamine oxidase, Suppl. 1 69
Amphetamines, plasma protein binding, 209
Anaesthesia and corneal indentation pulse, Suppl. 1, 52
Analgesics and monoamine uptake, Suppl. 1, 29
Andén, N.-E., 39 Suppl. 1, 30
Andersen, R. A., Suppl. 1, 31
Andersson, R. G. G. Suppl. 1, 32
Angiotensin blood plasma response, 184
– – pressure in nephrectomized rats, Suppl. 1, 83
Anoxia, protection by barbiturates, 203
Anticholinergics and apomorphine gnawing test, 1
– central and peripheral effect, 305
– human absorption, Suppl. 1, 14
– relative potency Suppl. 1, 84
Anticoagulant, toxicity of fluindarol, 425
Antidepressant, pharmacology of tricyclic compound, Suppl. 1 75
Antidepressive agents, pharmacology of thymoleptics, 241
Antifibrillatory effect of β-adrenergic blockers, 17
Antirheumatics, pharmacological properties, 249
Apomorphine gnawing test and anticholinergics, 1
Appelgren, Lars-Erik, Suppl. 1 33
Aquilonius, Sten-Magnus, Suppl. 1, 34 35
Arntzen, Finn Cato, 138
Arvela, P Suppl. 1, 36
Ascorbic acid distribution after benzanthrone, 499

ATP histamine release, Suppl. 1 43
Atropine determination on isolated organ, Suppl 1 84
– effect on oestrous behaviour Suppl. 1, 65
– inhibition of carbamoylcholine uptake, Suppl. 1, 51
– uptake by choroid plexus, Suppl. 1, 91

von Bahr Christer Suppl. 1, 13
Bakke, Olav M 28
Balwaal, J H., 338
Bárány Ernst, Suppl. 1, 37
Barbiturate, alcohol and meprobamate interaction, 334
– metabolism, 484
Barbiturates, protection against anoxia, 203
Barstad, J A. B Suppl. 1, 31
Bartoníček, V J 359
Beermann, Björn, Suppl. 1 14
Benzanthrone and ascorbic acid, 499
Bergendorff, Anders, Suppl 1, 38
Biliary excretion, quaternary and tertiary compounds, Suppl. 1, 78
Biotransformation, stereospecificity 445
Birtwhistle, R. D R., 425
Boman, Gunnar Suppl. 1, 15
Borgå, Olof, Suppl. 1, 15, 16
Boric acid, percutaneous absorption, 413
Boyd, E. M., 149
Bradykinin action, rat uterus, Suppl. 1, 41
Brisekl, Gudrun, 225 Suppl. 1 39
Briseld, Kjell, 124 138, Suppl. 1, 39
Broch J Ole Jacob Suppl. 1, 40
Broen Christensen, C., 215
Brun, A., 359
Bryn, Odd, Suppl. 1, 41
Butcher Sherrel G., 39 Suppl. 1, 30
Buzello, Walter 493
Buch, Horst, 493

Ca++ metabolic interaction, Suppl. 1, 32
Carbacholine, see carbamoylcholine
Carbamoylcholine uptake and atropine, Suppl. 1 51
Cardiac glycosides on isolated intestine, Suppl. 1, 39
Carlsson, S., Suppl. 1, 42
Catecholamine and protein synthesis, Suppl 1 82
– plasma protein binding, 209
Catecholamines and amphetamine metabolism, Suppl. 1 62
–, double isotope assay Suppl. 1, 74
– turnover 466
Catecholamine uptake and tyrosine, 378
Catechol-0-methyl transferase, fluorometric determination, Suppl 1 40
Cerium, effect on drug metabolism, Suppl. 1, 36
Chandra, Satya V 177
Chlorpromazine, histamine release, Suppl. 1, 48, 72
Choline acetyltransferase inhibitor Suppl. 1 34
– distribution and metabolism, Suppl. 1, 80
– in cerebrospinal fluid, Suppl. 1, 35
Cholinesterase in autonomic ganglia, 270
– inhibitors, barrier permeability Suppl. 1 31
Chorio-retinotoxic drugs on foetal eye, Suppl 1, 64
Christensen, C. Broen, see Broen Christensen, C.
Christoffersen, T Suppl. 1, 70
Circadian rhythm, ethanol effect, Suppl. 1, 77
Cloforex, pharmacokinetics, 391
Compound 48/80, ATP interaction, Suppl. 1, 44
Corrodi, Hans, 435
Corticosteroids in plasma fter thymoleptics, Suppl. 1 71
Cortisol, protein binding after indomethacin, Suppl. 1, 54
Cough induction in man, method, 103
51Cr-EDTA, reference substance in test meal, Suppl. 1 58
Cyclic 3'5'-AMP and adrenaline response, Suppl. 1 93
– – specific determination, Suppl. 1, 70
Cytochrome P-450 and desmethylimipramine, Suppl. 1 13

Dahlqvist, Rune, Suppl. 1, 43, 44
Decamethonium uptake by mouse kidney in vitro 192
– mouse liver in vitro, 215
– tubocurarine interaction, 370
Desmethylimipramine, metabolism in vitro, Suppl. 1 13

Diamant, Bertil, Suppl. 1, 43 44
2,4-Dichlorophenoxyacetic acid, effect on cell culture, Suppl. 1 57
Dicophane maximal tolerated doses, 149
– toxicity 149
Diethyldithiocarbamate and kinin system, Suppl. 1 88
Dihydrotestosterone distribution in mice, *Suppl. 1 33*
Dinitrophenol and ethanol metabolism, 113
Diphenylhydantoin, plasma concentration in man, Suppl 1 18, 19
– *protein binding, Suppl. 1 16*
– – – in newborn, Suppl. 1, 19
– see phenytoin
– side effects, Suppl. 1 18
Distribution of dihydrotestosterone, Suppl. *1 33*
Disulfiram and kinin system, 454 Suppl. 1, 88
L DOPA and blood pressure, Suppl. 1 50
– – receptor activity Suppl. 1 50
DOPA, error in turnover studies, 466
Dopamine stores, metatyramine protection of reserpine release, 39
Drug kinetics, theoretical considerations, Suppl. 1 73
– *metabolism, cerium interaction, Suppl.* 1, 36
2,4-D see 2,4-dichlorophenoxyacetic acid
Dyrud, Olav Kjell, 124 138

van Eeken, C. J 425
Elwin, Carl-Eric, 161
Emepronium, metabolic disposition, Suppl. 1 85
Emetic action, amino acid esters, 161
Engel, J Suppl. 1 30
Enhexymalum, see hexobarbital
Enibomalum, urinary excretion, 484
Enterocoating and absorption, 66
Eriksson, O Suppl. 1 42
Ethanol and kinin system, Suppl. 1 88
– effect on circadian rhythm, Suppl. 1 77
Ethanol-hexobarbital interaction, Suppl. 1, 90
Ethanol, kinin system, 454
– metabolism, dinitrophenols, 113

Felby S., 334
Fillion, Gilles, Suppl. 1 45 66
Fluindarol toxicity 425
Folic acid, effect on phenytoin metabolism, 265
Franck, K. F 241
Frankenberg, Lars, Suppl. 1, 34
Frankson, Gunhild, 209
Fredholm, Bertil B Suppl. 1, 46
Fredricson Overø K., 81
Frey H. H 477 Suppl. 1 47
Frisk Holmberg, Marianne Suppl. 1, 48
Fuxe, Kjell, 39

Gastric secretion, amino acid esters, 161
Gastrointestinal absorption in man, anticholinergics, Suppl. 1, 14
Guanethidine and sympathetic ganglia, 270
– autonomic ganglia catecholamines, 283
– – – cholinesterases, 283
– – – histology 283
– effect on sympathetic ganglia, Suppl. 1 56
Guldberg, Hans Cato, Suppl. 1, 40
Gunne, Lars-M., Suppl. 1 92

Haffner Jon F W Suppl. 1 49
Halothane metabolites, inhibition of metabolism, 327
– – toxicity 299
Hamberger Bertil, Suppl. 1 87
Hanngren, Åke, Suppl. 1, 15
Hansen, V 81
Hansson, Eskil, 435 Suppl. 1 78
Harthon, Lennart, 66
Helgeland, Kristen, Suppl. 1 57
Hellström, Kjell Suppl. 1 14
Henning, M., Suppl. 1, 50
Hermansen, Keld, 17 57
Hexamethonium uptake by mouse kidney in vitro, 192
– – mouse liver in vitro, 215
Hexobarbital-ethanol interaction, Suppl. 1 90
Hexobarbital isomers, relative effect, 493
5HIAA, synthesis and elimination in brain, Suppl. 1 86
Histamine, chlorpromazine, Suppl. 1 48
– release, Suppl. 1, 44
– – and chlorpromazine, Suppl. *1*, 72
– by ATP Suppl. 1 43

Hjalmarson, Ake, Suppl. 1 53
Holm, Jan, 192, 370, Suppl. 1, 51
Hyldberg, E., Suppl. 1, 54
5-Hydroxytryptamine and noradrenaline release, Suppl. 1 45
– cardiovascular response, Suppl. 1 66
– in mast cells, Suppl. 1, 58
– see serotonin
Hørven, Ivar Suppl. 1, 52

Indomethacin and cortisol, protein binding, Suppl. 1, 54
Isaksson, Olle, Suppl. 1, 53
Isoniazid, interaction with tuberculostatics, Suppl. 1, 15

Jacobsen, E., 203
Jacobsen, Sten, Suppl. 1, 41
Jalling, Birgitta, Suppl. 1, 19
Jansen, Jens Aas, Suppl. 1, 54
Jensen-Holm, Jens, 270, 283 Suppl. 1, 55, 56
Jensen, O Nygaard, 265
Johnsson, Birgitta, 49
Jonsen, Jon, Suppl. 1, 57
Joshi, N C., 499
Juul, Per 270, 283 Suppl. 1, 56
Jönsson, Lars-Erik, Suppl. 1, 92
Jörgensen, A., 81

Kallikrein, 124, 138
Kallikrein-trypsin inactivator 225
Kinetics of maximum blood concentration, Suppl. 1 73
Kinin releasing enzymes, 138
– system, disulfiram and ethanol, 454
– in rat plasma, 124
Kolberg, Jan, Suppl. 1, 57
Krebs cycle and ethanol, 113
Kristoffersen, John, Suppl. 1, 58
Kühl, N. T Suppl. 1, 56

Landmark, Knud, Suppl. 1, 49 59
Langslet, Asbjørn, 258, Suppl. 1, 60, 93
Larsson, Carin, Suppl. 1 61
Larsson, K. S., 249
Lassen, N 241
Lauren, H. H., Suppl. 1, 54
Leo 640, new tricyclic antidepressant, Suppl. 1, 75
Lewander Tommy Suppl. 1 62
Lillebell, G Suppl. 1, 63
Lin, F M 149
Lindquist, Nils Gunnar Suppl. 1, 64
Lindström, Leif Suppl. 1 65
Linch, Salvador Suppl. 1 45 66
Local anaesthetics, in vitro testing, Suppl. 1 63
Lund, Lars, Suppl. 1 18
Lunde, Per K. M., Suppl. 1, 16
Lundholm, Birgitta, Suppl. 1 34
Lundholm, L., Suppl. 1 32
Løkken, Per Suppl. 1 58
Laake, K., Suppl. 1 31

Magnussen, M. P Suppl. 1, 47
Magnusson, Göran, 435
Malmborg, Anne-Stina, Suppl. 1, 15
Malmnäs, Carl-Olof, Suppl. 1 67
Manganese chloride toxicity 177
Mast cell degranulation, Suppl. 1, 44
Mast cells, Suppl. 1, 43
– – 5-hydroxytryptamine, Suppl. 1, 58
Mattila, M. J Suppl. 1, 79
Maximum blood concentration kinetics, Suppl. 1, 73
Mephenesin intoxication and steroids, 145
Meprobamate, alcohol and barbiturate interaction, 334
poisoning, 334
Messenger theory and cyclic 3'5'-AMP Suppl. 1, 25
Metabolism, emepronium, Suppl. 1, 85
– in intestinal muscles, Suppl. 1, 32
– kinetics of trimethadione, 477
– of desmethylimipramine, Suppl. 1 13
– – carbomalum, 484
– – prostaglandin in tissues, Suppl. 1 61
– – terodiline, Suppl. 1 42
Metatyramine, protection against reserpine 39
Method, cough induction in man, 108
determination of COMT Suppl. 1 40
– double isotope assay of catecholamines, Suppl. 1 74
O-Methylation of phenols, 28
Methylatropine uptake by choroid plexus, Suppl. 1, 91
Methyltyrosine toxicity 435
Meyerson, Bengt J Suppl. 1, 65, 67 68

Mohme Lundholm, E., Suppl. 1, 32
Monoamine oxidase and amphetamine response, Suppl. 1, 69
– – inhibition, glyceryl trinitrate, 406
– uptake by blood platelets, Suppl. 1, 29
Monoamines and oestrous behaviour Suppl. 1, 65
– – testosterone interaction, Suppl. 1, 67
– uptake inhibition by analgesics, Suppl. 1, 29
Mulder D 425
Møller Nielsen, L, 241
Mørland, J Suppl. 1, 70

Nerve division, comparison with guanethidine, 270
Neuroleptics and noradrenaline uptake, Suppl. 1, 87
Nicotine, lethal brain concentrations, 233
– metabolism in vitro, 75
– toxicity in mice, 233
Nicotinic acid, oral absorption, 66
Nielsen, Gyda H., 413
Nielsen, I. Møller see Møller Nielsen, I.
Noradrenaline release by 5-hydroxytryptamine, Suppl. 1, 45
– uptake, thymoleptics and neuroleptics, Suppl. 1, 87
Nortriptyline, clinical pharmacology Suppl. 1, 11, 16, 20
– kinetic studies, Suppl. 1, 11
– plasma concentration in man, Suppl. 1, 20
– protein binding, Suppl. 1 16
– side effects, Suppl. 1 20
Nymark, M 241

Obianwu, Hopé Orakwue, Suppl. 1 69
Oanes, J B Suppl. 1, 70
Oubain induced arrhythmia mediation, 57
Overø, K. Fredricson, see Fredricson Overø K.

Palla, Asta, Suppl. 1 68
Pandya, K. P 499
Para-aminosalicylic acid, interaction with tuberculostatics, Suppl. 1, 15
Paraquat poisoning, 346
Parasympatholytics, see anticholinergics
Pedersen, V 241
Pakkarinen, Ahmo, Suppl. 1, 71
Pentaerythritol tetranicotinate, oral absorption, 66
Permeability of blood-retina barrier Suppl. 1 31
Persson, Henry 49
Persson, Torgny 378, 466
Perycit® see pentaerythritol tetranicotinate
Petersen, P V 241
Pharmacokinetics and amphetamine abuse, Suppl. 1 92
– of cloforex, 391
– – nortriptyline, Suppl. 1, 11
– – tuberculostatics, Suppl. 1, 15
Phenemalum, see phenobarbital
Phenobarbital, anticonvulsant effect, Suppl. 1, 47
Phenols, *O*-methylation, 28
Phenothiazine, reduction of Kefflux, Suppl. 1, 49
Phenothiazines, atrial excitability Suppl. 1, 59
– effect on heart, 258
Phenytoin metabolism, 265
– see diphenylhydantoin
– urinary clearance, 265
Pilocarpine and ovulation, Suppl. 1, 68
Plasma kinins, drug interaction, Suppl. 1 88
Poisoning, meprobamate, 334
– paraquat, 346
trichloroethylene, 359
Popóva, A. D Suppl. 1 72
Prilocaine biotransformation, 445
Prostaglandin metabolism in tissues, Suppl. 1 61
Protein binding, amphetamine, 209
– – catecholamines, 209
– – nortriptyline and diphenylhydantoin, Suppl. 1 16
Psychotropic drugs on grooming behaviour Suppl. 1 76

Quaternary ammonium compound, biliary excretion, Suppl. 1 78

Rane, Anders, Suppl. 1 19
Rasmussen, S., Suppl. 1, 73
Rauramo, E. Marjatta, Suppl. 1 86

Ravn-Jonsen, A., 484
Rentzhog, Leif, Suppl. 1 74
Reserpine, allyl alcohol hepatotoxicity 338
– and neostriatal dopamine stores, 39
Rifampicin, interaction with tuberculostatics, Suppl. 1, 15
Rohte, Oskar Suppl. 1, 75 76
Rosén, Anders, Suppl. 1, 14
Rosenberg, Per H., 299 327
Ross, S., 445
Rubensson, A., Suppl. 1, 50
Rydin, Erik, Suppl. 1 77
Ryrfeldt, Åke, 391, Suppl. 1, 78

Saarnivaara, Lalla, Suppl. 1, 29 79
Salicylates and inflammatory effect, 249
Schambye, P 113
Schael-Krüger J 1
Schuberth, J Suppl. 1, 35, 80, 81
Schulz, R., 477
Secher-Hansen, E., 97 102
Selye, Hans, 145
Serck-Hanssen, Guldborg, Suppl 1, 82
Serotonin, allyl alcohol hepatotoxicity 338
– see 5-hydroxytryptamine
Setekleiv Johannes, Suppl. 1, 49
Singh, G B 499
Sjöqvist, Folke, Suppl. 1, 15 87
Sjöstrand, Sven Erik, Suppl. 1 64
Skinhøj, Annette, 184, Suppl. 1, 83
Slanina, Premysl, 75, 233
Sleep mechanism, Suppl. 1, 23
Sorach, S A., Suppl. 1, 72
Sparf, B., Suppl. 1, 80, 81
Srinivasan, S., 338
Srinivasan, U 338
Srivastava, S. P 177
Stenslö, Karl-Erland, Suppl. 1, 34
Stålhandske, Torbjörn, 75, 233
Subcutaneous absorption, connective tissue, 102
– – of water and sucrose, 97
Sund, R. B., Suppl. 1, 84
Sundwall, A., Suppl. 1, 35, 42, 80, 81, 85, 89
Svedmyr Nils, 66
Sympathetic nerve degeneration, effect on smooth muscles, Suppl. 1 37
Syrdalen, Per Suppl. 1, 52

Tammisto, Tapani, 299 327
Terodiline, metabolic disposition, Suppl. 1 42
– metabolite identification, Suppl. 1, 89
Test meal reference substance, ^{51}Cr-EDTA, Suppl. 1 58
Testosterone, monoamine interaction, Suppl. 1, 67
Theophylline, metabolic and circulatory effect, Suppl. 1, 46
Thiophthalanes, metabolism, distribution and excretion, 81
Thymoleptic, thiophthalane compound, kinetics, 81
Thymoleptics and noradrenaline uptake, Suppl. 1, 87
– – plasma corticosteroids, Suppl. 1 71
– bicyclic, pharmacology 241
Thyroxine, allyl alcohol hepatotoxicity 338
Tissari, Anja H., Suppl. 1, 86
Tjeltveit, Olav Suppl. 1 57
Toluidine blue uptake and histamine release, Suppl. 1, 73
Tompsett, S. L., 346
Toxicity of manganese chloride, 177
Trasylol® assay method, 225
Trichloroethylene, neuropathology 359
– poisoning, 359
Tricyclic antidepressants, analgesia and pressor response, Suppl. 1, 79
Trifluoroethanol toxicity 299
Trimethadione, demethylation kinetics, 477
Tris activation of acetylcholinesterase, Suppl. 1 55
Tuberculostatics, pharmacokinetic interactions, Suppl. 1 15
Tubocurarine, interaction with decamethonium uptake, 370
Tuck, Dick, Suppl. 1 87
Tyrosine and catecholamine uptake, 378

Ullberg, Sven, Suppl. 1, 64
Ursin, Reidun, Suppl. 1, 23
Uthne, K., Suppl. 1 42, 85
Uvnäs, Börje, Suppl. 1 38, 72

Vendsborg, P B., 113
Venneröd, Anna Marie, 454, Suppl. 1 88
Vessman, J Suppl. 1, 42, 85 89

Wahlström, Göran, 493 Suppl. 1, 77 90
Waldeck, Bertil, 406, 466
Wilhjelm, Bendt J 203
Winbladh, Birger Suppl. 1, 91
Winther *Finn Ø* 108

Änggård, Erik, 209 Suppl. 1, 61, 92

Øie, Svein, 124
Øye, Ivar Suppl. 1 25, 60, 70, 93

Åberg, G 249
Åkerman, B., 445
Åsberg, Marie, Suppl. 1, 20

Acta pharmacol. et toxicol. 1970 28 1–16.

From the Department of Pharmacology and Toxicology The Royal Veterinary and Agricultural College and The Danish Defence Research Board, Copenhagen, Denmark

Central Effects of Anticholinergic Drugs Measured by the Apomorphine Gnawing Test in Mice

By

J. Scheel-Krüger*

(Received April 28, 1969)

Abstract. Apomorphine in doses ranging from 10 up to 60 mg/kg given subcutaneously to mice induces only weak gnawing behaviour and 10 mg/kg was without effect. Th addition of anticholinergic drugs given 15 min. before 10 mg/kg apomorphine potentiated the gnawing behaviour 9 tertiary and 3 quaternary drugs were tested. Among these some quinuclidinylesters, scopolamine and benztropine were found to be very active compared with atropine. The quaternary compounds showed much weaker activity than the corresponding tertiary anal ogues. The gnawing activity produced by atropine plus apomorphine was only weakly antagonized by the apomorphine antagonistic drug spiramide; physostigmine showed a better inhibitory effect and combined treatment with spiramide and physostigmine gave a pronounced antagonistic effect. Since the apomorphine gnawing behaviour is most probably related to an interaction with central dopamine receptors, these findings suggest there is a central counter balancing dopaminergic-cholinergic system.

Key-words: Apomorphine – anticholinergic drugs.

In the psychopharmacological research for the evaluation of antipsychotic drugs there has been particular interest in drugs showing antagonism against amphetamine or apomorphine induced central excitation, which in rats and mice consists of a stereotyped behaviour with continuous sniffing, licking and gnawing.

The correlation between the clinical effect on schizophrenic symptoms and the anti-stereotypic effect in animal experiments is reported to be very high for antipsychotic drugs (JANSSEN *et al.* 1960 1965 & 1967 RANDRUP & MUNKVAD 1965 MUNKVAD & RANDRUP 1966 MUNKVAD *et al.* 1968 VAN ROSSUM 1966)

* Present address: Central Laboratory Dept. E, Sct. Hans Hospital, DK-2100 Roskilde, Denmark.

Wahlström, Göran, 493, Suppl. 1 77 90
Waldeck, Bertil 406, 466
Wilhjelm, Bendt J 203
Winbladh, Birger Suppl. 1 91
Winther Finn Ø 108

Änggård, Erik, 209 Suppl. 1 61 92

Öie, Svein, 124
Øye, Ivar Suppl 1 25, 60, 70, 93

Åberg, G 249
Åkerman, B., 445
Åsberg, Marie, Suppl. 1, 20

Counting of the gnawing intensity

The intensity of th gnawing behaviour was estimated by means of a wire netting (14 × 22 cm) provided with 11 × 8 = 198 squares. The wire netting was fixed over the corrugated paper and the numbers of squares, through which biting marks in the corrugate could be observed, were counted. Since 3 papers were used for the estimation of th biting intensity the theoretical maximum value was 3 × 198 = 594 counts. This way of estimating the biting intensity is different from the methods used by THIER & SCHRAMM (1962) and PEDERSEN (1967 & 1968) who only assess the biting intensity from an area corresponding to 10 % of the corrugated paper

Experimental schedule

1 A quantitative determination of the apomorphine potentiation as shown by the combined treatment of anticholinergic drugs and apomorphine was performed. – The following tertiary anti-cholinergic drugs were used. Atropine sulphate scopolamine hydrobromide; benactyzine hydrochloride; benztropine hydrobromide, caramiphen hydrochloride; N methyl-4-piperidyl-1-phenylcyclopentane-carboxylate hydrochloride 3-quinuclidinylbenzilate hydrochloride; 3-quinuclidinyl-1-phenylcyclopentane-carboxylate hydrochloride, and 3-quinuclidinyl-diphenylacetate hydrochloride. The quaternary anticholinergic drugs were N-methyl-3-quinuclidinyl benzilate bromide; N-methyl-atropine nitrate, and N-methylscopolamine nitrate.

In this procedure the anticholinergic drugs were injected intraperitoneally 15 min. before a fixed dose of 10 mg/kg pomorphine hydrochloride, given subcutaneously Twenty minutes after apomorphine the gnawing intensity was determined. In order to equalize any possibl variations in the intensity of gnawing behaviour from day to day th measurements of each test drug were extended for a period of 14–30 days. A control group receiving 10 mg/kg apomorphine only was also included on each test day Furthermore, these experiments were only performed in the forenoon, since preliminary experiments showed that the gnawing intensity was decreased by about 25 % in the afternoon.

2 The inhibition of the atropine-apomorphine induced gnawing was investigated in the afternoon. In these studies all the mice received a combined treatment of apomorphine (10 mg/kg subcutaneously) given 15 min. fter atropine (20 mg/kg intraperitoneally). Spiramide (R5808), which is reported to be a highly potent amphetamine and apomorphine antagonist (JANSSEN *et al* 1965 VAN ROSSUM 1966) was given intraperitoneally 1 hour before atropine in various doses ranging from 0.1 20 mg/kg. Physostigmine salicylate was tested in doses of 0.1 0.5 and 1 0 mg/kg given intraperitoneally simultaneously with apomorphine. In all these experiments the gnawing behaviour was estimated during 40 min. starting 20 min. after the administration of apomorphine.

Determination of the ED50 values of the anticholinergics.

Each of the anticholinergic drugs was tested in 8–10 doses in order to determine the dose interval during which gnawing was increased from minimum to maximum intensity Within this dose interval, th experiments were performed with at least 5 doses, each of which was repeated 4–6 times. The mean values of the gnawing counts corresponding to these doses were calculated and expressed in per cent of maximum value fixed to 300 counts. This theoretical maximum value is approximately 5–10 % bove the highest values obtained in the experiments with all the anticholinergic drugs. The values for the gnawing intensity representing the doses from which the gnawing increased from zero to the maximal intensity were drawn on logarithmic probability paper (Copyright AGF2109). Th linear relationships between doses (logarithmic scale)

and the values for the gnawing (probability scale) were calculated and tested according to the least square method (DAVIES 1958). The ED50 is thus the dose corresponding to a gnawing intensity of 150 counts.

Results

Behavioural effects of apomorphine

A central stimulation was induced by apomorphine given alone in doses of 10 15 20, 30 40 and 60 mg/kg subcutaneously Following the administration of 10 mg/kg continuous sniffing, increased locomotion and rearing ("standing-up on hind legs) were observed. Normal behavioural elements such as eating, drinking and grooming were absent.

Increased stimulation was seen with 15 and 20 mg/kg. The rearing behaviour extended to a marked climbing up of the corrugated paper placed towards the side. An exaggerated central excitation was seen with doses of 30 and 40 mg/kg. The rearing and climbing-up" led to characteristic attempts to escape from the cage. A good deal of jumping in the cage was also observed. Some deaths occurred with doses of 40 and 60 mg/kg. This be

Table 1

Apomorphine-induced gnawing behaviour in mice

Apomorphine hydrochloride was given subcutaneously to mice using 3 mice in each experiment. The gnawing behaviour was estimated for 40 min. starting 20 min. after pomorphine. (For further details, see Methods" section)

Apomorphine hydrochloride. Doses in mg/kg	Number of *experiments*	Gnawing intensity*
10	10	0
15	5	0, 6, 9 17 24
20	10	0 (6 experiments), 7 7 33 78
30	15	0 (9 experiments), 16, 29 30, 32, 37 100
40	8	0 (*5 experiments*), 2, 7 20
60	5	0 (2 experiments), 12, 12, 42

Expressed in % of a maximal value of 300 used in the experiments with pomorphine and anticholinergic drugs.

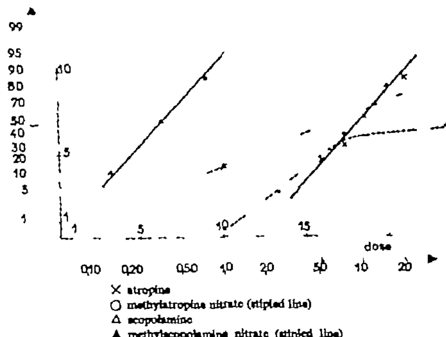


Fig. 1 The gnawing intensity is drawn on a probability scale and the doses f th anticholinergic drugs on a logarithmic scale (abscissa). 10 mg/kg apomorphine was given 15 min. after the anticholinergic drugs. The stipled system of co-ordinates is used for the calculation of the regression coefficients.

havioural excitation lasted for about 45–60 min. for the lowest doses and up to approximately 90 min. for the highest doses. The gnawing behaviour was particularly investigated in experiments with 3 mice per cage. The results are summarized in table 1

A dose of 10 mg/kg apomorphine already induced chewing movements of the jaws, but biting of the applied corrugated paper in the cage did not occur Higher doses of apomorphine sometimes induced gnawing of the paper However, the counts for the gnawing intensity were most variable and not reproducible in these experiments.

Combination of anticholinergics and apomorphine

The results (graphically summarized in figs. 1 2 & 3) showed that a potentiation of the gnawing behaviour was obtained with all of the 12 anticholinergic drugs (tertiary and quaternary compounds) used. Approximately the same maximal value for the gnawing intensity was obtained for all the tertiary compounds. Even much higher doses of the anticholinergic drugs

(not shown in the figures) did not induce a higher gnawing intensity The gnawing behaviour as determined by the counts on the paper placed towards the side was high for all these drug combinations.

The locomotion, rearing and sniffing were seen very shortly after the apomorphine injection in this combined drug treatment. Gnawing occurred about 10 min. after apomorphine and was almost continuous after another 10 min.

However if the tertiary anticholinergic drugs were given alone without apomorphine, they induced as observed by gross inspection, increased locomotion and sniffing but no gnawing of the corrugated paper The quaternary anticholinergic drugs (methylatropine, methylscopolamine and methylquinuclidinyl benzilate) given alone, however did not induce any significant behavioural stimulation.

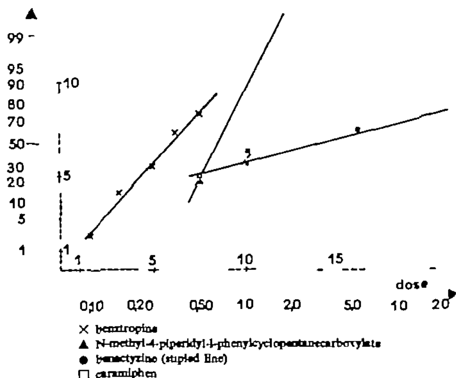


Fig. 2. The gnawing intensity is drawn on probability scale and the doses of the anticholinergic drugs on a logarithmic scale (abscissa). 10 mg/kg apomorphine was given 15 min. after the anticholinergic drugs. The stipled system of co-ordinates is used for the calculation of the regression coefficients.

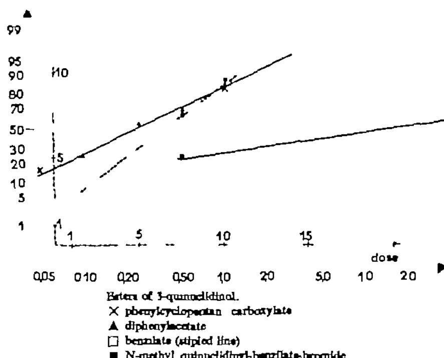


Fig. 3 Th gnawing intensity is drawn on probability scale and th doses of the anticholinergic drugs on a logarithmic scale (abscissa). 10 mg/kg apomorphine was given 15 min. after the anticholinergic drugs. The stipled system of co-ordinates is used for the calculation of th regression coefficients.

Table 2 summarizes the values for the regression coefficients which correspond to the slopes of the regression lines in the system of coordinates drawn with stipled lines in figs. 1 2 and 3 Similar slope values were obtained for the following classical anticholinergic drugs: atropine, scopolamine, benactyzine and benztropine. Scopolamine and benztropine were found to be 26 and 27 times, respectively more active than atropine. Benactyzine was 7.3 times more active.

The slope values for all the other anticholinergics were found to be significantly different from the values for atropine.

The very low ED50 values for the 3-quinuclidinyl-esters suggest a very high central activity for these drugs. However the values for the relative potency (indicated in brackets) compared with atropine are to be taken with some reservation, since the slope values deviate significantly

By gross inspection the behavioural stimulation obtained with the quater

Table 2

Potentiation of apomorphine-induced gnawing by anticholinergic drugs

The anticholinergics in various doses were injected 15 min. before 10 mg/kg pomorphine. For details of procedure, see "Methods section. The regression values correspond to the slope of the regression lines in the system of co-ordinates drawn with stipled lines in figs. 1 2 & 3

Anticholinergics	Regression coefficients ± S.E.M (95 % confidence limits)	ED50 (mg/kg)	Relative activity (ED50 atropine/ED50)
Atropine	1 13 ± 0.13 (0 73 – 1 53)	8.8	1.00
Benactyzine	1.21 ± 0.14 (1.07 – 1.35)*	1.2	7.3
Scopolamine	1 07 ± 0 093 (0.77 – 1.37)*	0.34	26
Benztropine	1 11 ± 0.068 (0 89 – 1.33)*	0.33	27
Caramiphen	0.26 ± 0.050 (0 12 – 0 40)**	3.0	(2.9)
N-methyl-4-piperidyl-phenylcyclopentane carboxylate	2.11 ± 0.29 (0.85 – 3.37)*	0.64	(14)
3-quinuclidinyl-benzilate	0 76 ± 0.069 (0.57 – 0.95)*	0.37	(24)
3-quinuclidinyl-phenylcyclopentane carboxylat	0.48 ± 0.055 (0.31 – 0.65)	0.24	(37)
3-quinuclidinyl-diphenyl acetate	0.48 ± 0.034 (0.38 – 0.58)**	0.24	(37)
N-methyl-atropine nitrate	– –	–	–
N-methylscopolamine nitrate	0.39 ± 0 077 (0.18 – 0.60)*	5 4	(1.6)
N-methyl-quinuclidinyl benzilate bromide	0.14 ± 0.071 (0.00 – 0.31)	13	(0.68)

No significant difference of the regression coefficient from the result with atropine (P > 0 05).

** The regression coefficient differs significantly from atropine (P < 0.05)· the relative activity of these compounds compared with th result of atropine is therefore indicated in brackets.

nary compounds and apomorphine was reminiscent of the effect of the tertiary compounds. The excitation was, however more moderate with methyl atropine and a linear relationship between dose response was not obtained. A linear relationship was obtained for methyl-quinuclidinyl benzilate and methylscopolamine with slopo values significant from zero

Inhibition of the atropine-apomorphine induced gnawing behaviour

The gnawing behaviour in all of these studies was produced by a combined drug treatment with atropine and apomorphine given in fixed doses of 20 and 10 mg/kg, respectively The results are summarized in table 3

This gnawing behaviour was significantly antagonized by physostigmine in doses of 0.5 and 1.0 mg/kg. The locomotion, rearings and sniffing were repressed by a dose of 1 0 mg/kg. However neurological disturbances such as tremor and ataxia were observed in a few mice for short periods. No

Table 3

Inhibition of the atropine-apomorphine induced gnawing behaviour

All mice were treated with 20 mg/kg atropine and 10 mg/kg apomorphine. Spiramide was given 1 hour before atropane. Apomorphine and physostigmine were given 15 min. after atropine. Each value for the gnawing behaviour represents th mean ± standard error of the mean of 5 experiments, each performed with 3 mace. However th control group receiving atropine-apomorphine only represents 9 experiments

Further drug treatment (doses in mg/kg)		Counts for gnawing behaviour ± S.E.M	
Spiramid	Physostigmine		
0	0	210 ± 14	(Control Group)
0	0.10	174 ± 28	
0	0.50	111 ± 14	$P < 0.001$
0	1.0	63 ± 10*	$P < 0.001$
0.10	0	250 ± [illegible]	
0.50	0	184 ± 26	
1.00	0	174 ± 25	
2.00	0	140 ± 20*	$P < 0.02$
5.00	0	76 ± 36	$P < 0.01$
20.0	0	5 ± 7*	$P < 0.001$
0.10	0.10	118 ± 24	$P < 0.01$
0.50	0.10	131 ± 31	$P < 0.02$
1.0	0.10	104 ± 29*	$P < 0.01$

*These results differ significantly from the control group.

Table 2

Potentiation of pomorphine-induced gnawing by anticholinergic drugs

The anticholinergics in various doses were injected 15 min. before 10 mg/kg apomorphine. For details of procedure, see "Methods" section. The regression values correspond to the slope of the regression lines in the system of co-ordinates drawn with stipled lines in figs. 1 2 & 3

Anticholinergics	Regression coefficients ± S.E.M (95 % confidence limits)	ED50 (mg/kg)	Relative activity (ED50 atropine/ED50)
Atropine	1 13 ± 0.13 (0 73 – 1.53)	8 8	1.00
Benactyzine	1.21 ± 0.14 (1.07 – 1.35)*	1.2	7.3
Scopolamine	1.07 ± 0.093 (0.77 – 1.37)*	0.34	26
Benztropine	1 11 ± 0.068 (0.89 – 1.33)*	0.33	27
Caramiphen	0.26 ± 0.050 (0.12 – 0.40)**	3.0	(2.9)
N-methyl-4-piperidyl-phenylcyclopentane carboxylate	2.11 ± 0.29 (0 85 – 3.37)**	0.64	(14)
3-quinuclidinyl-benzilat	0.76 ± 0.069 (0.57 – 0.95)**	0 37	(24)
3-quinuclidinyl-phenylcyclopentane carboxylate	0 48 ± 0.055 (0.31 – 0 65)*	0.24	(37)
3-quinuclidinyl-diphenyl acetate	0.48 ± 0.034 (0.38 – 0.58)*	0.24	(37)
N-methyl-atropine nitrate	– –	–	–
N-methylscopolamine nitrate	*0.39 ± 0 077 (0.18 – 0.60)**	5 4	(1.6)
N-methyl-quinuclidinyl benzilate bromide	0.14 ± 0.071 (0.00 – 0.31)	13	(0.68)

No significant difference of the regression coefficient from the result with atropine (P > 0.05).

The regression coefficient differs significantly *from atropine (P < 0.05); the relative activity of these compounds compared with the result* of tropine is therefore indicated in brackets.

methylpiperidyl-phenylcyclopentane carboxylate showed activities which were respectively 2.9 7.3 and 14 times greater as compared with atropine. The synergistic effect with apomorphine shown by the quaternary amines was unexpected, since these drugs pass the blood-brain barrier only to a very small extent (GOODMAN & GILMAN 1965). However the response obtained with methylscopolamine and methylquinuclidinyl benzilate occurred at relatively high dose levels which were at least 10 times higher than those of the tertiary amines. With methylatropine and methylquinuclidinyl benzilate in extremely high doses, values for the gnawing intensities were obtained which were only about 50-60 / of those for the tertiary amines. However other tests in common use for measurement of the central activity have revealed similar but weaker effect of the quaternary anticholinergic drugs compared with the tertiary analogues. Thus with respect to the increase of the spontaneous locomotor activity in mice, methylscopolamine was only 1/100 as active as scopolamine (HARRIS 1961). Methylatropine too showed weaker activity than atropine in mice (MYERS *et al.* 1964) and in rats (PRADHAN & ROTH 1968) Methylatropine has weak atropine-like effects as measured by EEG activity (POUL DAVID *et al* 1960 CARLTON & HOROVITZ 1961 RIEHL *et al.* 1962) and also in tests on the potentiation of the amphetamine effect in conditioned avoidance procedures (CARLTON & DIDAMO 1961 SCHECKEL & BOFF 1964)

All the anticholinergic drugs used in this gnawing test have been tested alone and also in combination with the oxime reactivator TMB-4 against the toxicity in mice with the acetylcholinesterase inhibitor paraoxone (KARLOG 1967). The procedure has been used by RIBBENTROP & SCHAUMANN (1965) as a test for measuring the central potency of anticholinergic drugs. In agreement with this the results obtained in the gnawing test correspond to the activity obtained against the paraoxone poisoning (KARLOG unpublished).

It was stated in the introduction that the apomorphine or amphetamine induced gnawing behaviour is most probably dependent on an interaction with dopamine receptors in the corpus striatum. The anticholinergic drugs given even in very high doses did not produce the compulsive gnawing behaviour but the combined treatment with anticholinergic drugs and a small ineffective dose of apomorphine produced a very high gnawing intensity These results allow of the conclusion that the activity of a cholinergic inhibitory system in the central nervous system plays an important role in modifying the activity of a dopaminergic system. Inhibition of the cholinergic system with anticholinergic drugs uncovers the adrenergic dominance as is evident from the apomorphine induced gnawing.

The nature and role of the cholinergic system acting in this proposed cholinergic-dopaminergic counter balancing system, was further investigated. Activation of the cholinergic system produced by physostigmine in agreement

with the findings of PEDERSEN (1967) showed a significant inhibition of the atropine apomorphine induced gnawing.

In addition, other central activities produced by adrenergic drugs are antagonized by central cholinergic drugs e. g. physostigmine, tremorine, oxotremorine, and arecoline.

These central adrenergic activities concerned the amphetamine induced stereotypies in rats (SCHELKUNOV 1964 & 1967 ARNFRED & RANDRUP 1968) amphetamine disturbances of conditioned reflexes of rats in a labyrinth (SCHELKUNOV 1963 & 1967) amphetamine toxicity in aggregated mice (MENNEAR 1965) and amphetamine and methamphetamine increased motility in mice (MENNEAR 1965 GALAMBOS *et al.* 1967 TRIPOD 1952).

As another possibility for the inhibition of the atropine-apomorphine induced gnawing, the activity of spiramide was investigated. Among the neuroleptics spiramide is reported to be the most potent and specific antagonist of the apomorphine and amphetamine induced gnawing behaviour in rats (JANSSEN *et al.* 1965 & 1967 VAN ROSSUM 1966) The experiments showed, however that spiramide in this situation only produced a significant inhibition of the gnawing behaviour when given in high and massive doses.

This result agrees with other published evidence which has revealed that anticholinergic drugs antagonize several behavioural effects considered to be highly characteristic for neuroleptic drugs catalepsy and antiamphetamine actions of neuroleptics (MORPURGO 1962 MORPURGO & THEOBALD 1964 LESLIE & MAXWELL 1964) PEDERSEN (1967) found that a small dose of flupentixol (a very potent neuroleptic drug) was without significant effect against the gnawing in mice induced by apomorphine in combination with anticholinergics. MORPURGO & THEOBALD (1964), SCHAUMANN & KURBJUWEIT (1961) and TAESCHLER *et al.* (1962) found that the disturbances induced by neuroleptics of a conditioned avoidance response in rats were antagonized by anticholinergic drugs. All these results thus indicate that the activity of a cholinergic inhibitory system in the central nervous system plays an important role in the actions of neuroleptic drugs.

Cholinergic agents in agreement with this suggestion show some actions which are considered as characteristic for neuroleptic drugs thus arecoline pilocarpine, tremorine, nicotine and paraoxone are reported to induce both hypothermia and a cataleptic state in mice (ZETLER 1968). PFEIFFER & JENNY (1957) HEALY & JENNY (1959) and CHALMERS & ERICKSON (1964) have described a blocking effect of centrally acting cholinergics in a conditioned avoidance response in rats PFEIFFER & JENNY (1957) furthermore found a short lasting improvement in schizophrenic patients after arecoline

A significant synergistic action of the neuroleptic agent spiramide and physostigmine against the atropine-apomorphine induced gnawing was found. Other findings with cross-potentiation between cholinergics and neuroleptics

are described in the tests consisting of. Discrete avoidance behaviour in rats (GOLBERG & JOHNSON 1964), hexobarbital sleeping time amphetamine increased toxicity in aggregated mice and amphetamine induced motility (PROCTOR *et al.* 1964). Increased toxicity of the anticholinesterase drug parathion (nitrostigminum NFN) by chlorpromazine is also reported in rats (GAINES 1962) as well as the potentiation of the bulbocapnine induced catalepsy with cholinergic drugs (ZETLER *et al.* 1960)

Acknowledgment

The author is grateful to H. Westenbæk Hansen, The Danish Defence Research Board, Copenhagen, for the synthesis of most of the anticholinergic drugs.

REFERENCES

Amsler C Beiträge zur Pharmacologie des Gehirns. *Arch. exp Path Pharm.* 1923 97 1 14

Andén, N.-E., A. Rubenson, K. Fuxe & T Hokfelt Evidence for dopamine receptor stimulation by apomorphine. *J Pharm. Pharmacol* 1967 19 627–629

Arnfred, T. & A. Randrup Cholinergic mechanism in brain inhibiting amphetamine-induced stereotyped behaviour *Acta pharmacol et toxicol* 1968, 26 384–394.

Carlton, P L. & P Didamo Augmentation of the behavioural effects of amphetamine by atropine. *J Pharmacol Exper Therap* 1961 134 91–96.

Carlton, P L. & Z. P Horovitz. Behavioural and EEG effects of atropine and methyl atropine (Paper read at the meetings of the Eastern Psychological Association 1961).

Chalmers, R. K. & C. K. Erickson. Central cholinergic blockade of the conditioned avoidance response in rats. *Psych pharmacologia* 1964 6, 31–41

Davies, O L. *Statistical methods in research and production* 1958, p. 51–68, p. 150–207 Oliver and Boyd, London.

Ernst, A. M.. Mode of action of apomorphine and dexamphetamine in gnawing compulsion in rats. *Psychopharmacologia* 1967 10 316–323

Ernst, A. M. & P G Smelik: Site of action of dopamine and apomorphine on compulsive gnawing behaviour in rats. *Experientia* 1966, 22, 837–838.

Fog, R., A. Randrup & H. Pakkenberg: *Amines in the corpus striatum associated with the effects of both amphetamine and antipsychotic drugs. Excerpta Medica Intern Congress Series no 150* Proc IV World Congress of Psychiatry 1966, 2580–2582.

Fog, R., A. Randrup & H. Pakkenberg: Aminergic mechanisms in corpus striatum and amphetamine-induced stereotyped behaviour *Psychopharmacologia* 1967 11 179–183

Gaines, T B. Poisoning by organo phosphorus pesticides potentiated by phenothiazine derivates. *Science* 1963, 138, 1260–1261

Galambos, E A. K. Pfeifer L. György & J Molnar: Study on the excitation induced by amphetamine, cocain and α-methyltryptamine. *Psychopharmacologia* 1967 11, 122 129

Goldberg, M E. & H. E. Johnson. Behavioural effects of a cholinergic stimulant in combination with various psychotherapeutic agents. *J Pharmacol Exper Therap.* 1964 145 367–372.

Goodman, L. S. & A. Gilman. *The pharmacological basis of therapeutics* 1965 3rd Ed., p. 533 536, Macmillan Company N Y

Harris, L. S., Th effect of various anti-cholinergics on spontaneous activity of mice. *Fed. Proc* 1961 20 395

Healy S. T & J E. H. Jenny Drug antagonists to arecoline inhibition of the conditioned response. *Fed Proc.* 1959 18, 400.

Hornykiewicz, O Dopamine (3-hydroxytyramine) and brain function. *Pharmacol Rev* 1966, 18, 925–964

Hornykiewicz, O Gegenwärtiger Stand der biochemisch-pharmakologischen Erforschung des extrapyramidal-motorischen Systems. *Pharmakopsychiat* (Stuttgart, Thieme) 1968, 1, 6–17

Janssen, P A. J C. J E. Niemegeers & A. H. M. Jageneau. Apomorphine antagonism in rats. *Arzneimittel-Forschung* 1960 10 1003–1005

Janssen, P A. J C. J E. Niemegeers & K. H. L. Schellekens Is it possible to predict the clinical effects of neuroleptic drugs (Major Tranquillizers) from animal data? *Arzneimittel-Forschung* 1965 15 104–117

Janssen, P A. J C. J E. Niemegeers, K. H. L. Schellekens & F M. Lenaerts. Is it possible t predict the clinical effects of neuroleptic drugs (Major Tranquillizers) from animal data? *Arzneimittel-Forschung* 1967 17 841–854

Karlog, O Atropin substitutes alone and in combination with TMB-4 used as protection against Paraoxone intoxication in mice. *Acta pharmacol. et toxicol.* 1967 25, suppl. 4 76.

Leslie, G. B & D. R. Maxwell Some pharmacological properties of thioproperazine and their modification by anti-parkinson drugs. *Brit J Pharmacol* 1964 22, 301 317

Mennear J H. Interactions between central cholinergic agents and amphetamine in mice. *Psychopharmacologia* 1965 7 107–114

Morpurgo, C. Effects of antiparkinson drugs on a phenothiazine induced catatonic reaction. *Arch int Pharmacodyn.* 1962, 137 84–90.

Morpurgo, C. & W Theobald. Influence f antiparkinson drugs and amphetamine on some pharmacological effects of phenothiazine derivates used as neuroleptics. *Psych pharmacologia* 1964 6 178–191

Munkvad, I & A. Randrup The persistance of amphetamine stereotypies of rats in spite of strong sedation. *Acta psychiat scand* 1966, 42, suppl. 191 178–187

Munkvad, I., H. Pakkenberg & A. Randrup Aminergic systems in basal ganglia associated with stereotyped hyperactive behaviour and catalepsy *Brain* Behav Evol. 1968, 1 89–100.

Myers, B K. H. Roberts, R. H. Riciputi & E. F Domino Some effects of muscarinic cholinergic blocking drugs on behaviour and the electro-corticogram. *Psychopharmacologia* 1964, 5 289–300.

Parkes, M. W An examination of central actions characteristic of scopolamine: Comparison of central and peripheral activity in scopolamine, atropine and some synthetic basic esters. *Psychopharmacologia* 1965 7 1 19

Pedersen, V Potentiation of apomorphine effect (Compulsive gnawing behaviour) in mice. *Acta pharmacol. et toxicol* 1967 25 suppl. 4, 63

Pedersen, V.. Role of catecholamines in compulsive gnawing behaviour in mice. *Brit J Pharmacol.* 1968, 34 219

Pfeiffer C. C. & J E. H. Jenny· The inhibition of th conditioned response and the counteraction of schizophrenia by muscarinic stimulation of the brain. *Ann. N Y Acad. Sci.* 1957 66, 753–764

Pletscher A. & M. Da Prada. Mechanism of action of neuroleptics. *Excerpta Medica Intern. Congress Series 129, Proc. 5th Intern. Congress Collegium Intern. Neuro-*psychopharmacologium, Washington 1966, 304–310.

Poul-David, J J L. Riehl & K. R. Unna Quantification of effects of depressant drugs on EEG activation response. *J Pharmacol Exper Therap* 1960, 129 69–74.

Da Prada, M. & A. Pletscher· On the mechanism of chlorpromazine induced changes of cerebral homovanillic acid levels. *J Pharm. Pharmacol.* 1966, 18, 628–630.

Pradhan, S. N & T Roth Comparative behavioural effects of several anticholinergic agents in rats. *Psychopharmacologia* 1968, 12, 358–366.

Proctor C. D S. A. Ridlon, J J Fudema & V G Prabhu Extension of tranquillizer action by anticholinesterases. *Toxicol and Appl Pharmacol* 1964 6 1–8.

Randall, L. O W M Benson & P L. Stefko· Spasmolytic action of bicyclic basic alcohol esters. *J Pharmacol Exper Ther* 1952, 104 284–290.

Randrup, A. & I. Munkvad Special antagonism of amphetamine induced abnormal behaviour *Psychopharmacologia 1965 7 416–422.*

Randrup, A. & I. Munkvad. Behavioural stereotypies induced by pharmacological agents. *Pharmakopsychiat.* (Stuttgart, Thieme) 1968, 1, 18–26.

Ribbentrop, A. & W Schaumann. Zentrale anticholinerge Wirksamkeit und Spezifität einiger Derivat Granatans. *Arzneimittel-Forschung* 1965 15, 857–862.

Riehl, J L., J Poul-David & K. R. Unna. Comparison of the effects of arecoline and muscarine on the central nervous system. *Int J Neuropharmacol* 1962, 1 393–401

Roos, B. E. Decrease in homovanillic acid as evidence for dopamine receptor stimulation by apomorphine in the neostriatum of the rat. *J Pharm. Pharmacol.* 1969 21 263–264.

Van Rossum, J M The significance of dopamine-receptor blockade for th action of neuroleptic drugs. *Excerpta Medica Intern. Series No 129* Proc. 5th int Congress Collegium Intern. Neuropsychopharmacologium, Washington 1966, 321 329

Schaumann, W & H. G Kurbjuweit: Beeinflussung verschiedener Wirkungen von Thiopropazat durch ein zentrales Stimulans. *Arzneimittel-Forschung* 1961 11, 343–350.

Schallek, W & T H. F Smith: Electroencephalographic analysis of side effects of spasmolytic drugs. *J Pharm. Exper Ther* 1952, 104 291–298.

Scheckel, C. L. & E. Boff Behavioural effects of interacting imipramine and other drugs with d-amphetamine, cocaine, and tetrabenazine. *Psychopharmacologia* 1964, 5, 198–208

Schelkunov E. L. Integrated effect of psychotropic drugs on the balance of cholino-adreno- and serotoninergic processes in the brain as a basis of their gross behavioral and therapeutic actions. *Activ nerv sup.* (Praha) 1967 8, 207–217

Schelkunov E. L. The technique of phenamin stereotypy for evaluating th effect produced by remedial gents on the central adrenergic processes. *Pharmacol Toxicol.* (russ.) 1964, 27 628–633

Taeschler M., H. Weidman & A. Cerletti. Zur Pharmakologie von Proavid, einem neuen zentralen Anticholinergicum. *Schweiz. Med. Wschr* 1962, 90 1542–1545

Tber L. & H. Schramm. Apomorphine-synergismus (Zwangsnagen bei Mäusen) als Test zur Differenzierung psychotroper Substanzen. *Arch int Pharmacodyn.* 1962, 138, 302 310.

Tripod, J Beeinflussung der zentral-erregenden Wirkung von Weckaminen durch Pharmaka mit spezifischer Wirkung auf das autonome Nervensystem. *Helv Physiol Acta* 1952, 10 403–412.

Zetler G Cataleptic state and hypothermia in mice, caused by central cholinergic stimulation and antagonized by anticholinergic and antidepressant drugs. *Int J Neuropharmacol* 1968, 7 325–335

Zetler G K. Mahler & F Daniel. Versuche zu einer pharmakologischen Differenzierung kataleptischer Wirkungen. *Arch exp Path. Pharmakol* 1960, 238, 486–501

Acta pharmacol. et toxicol. 1970 28 17–27

From the Research Division of Pharmacia AS,
Vanløse, Copenhagen, Denmark

Antifibrillatory Effect of Some β-Adrenergic Receptor Blocking Agents Determined by a New Test Procedure in Mice

By

Keld Hermansen

(Received July 9 1969)

Abstract: Mice which were exposed to high concentrations of methylchloroform (1 1,1, trichloroethane) vapour died in cardiac fibrillation. Pretreatment 30 min. before the administration of methylchloroform gave the following antifibrillatory ED50's expressed in mg/kg intraperitoneally: β-adrenergic blocking agents Propranolol 1 7 (+)-propranolol 45 (−)-propranolol 0 6 1-(isopropylamino)-3-(o-phenoxyphenoxy)-2-propanol, HCl (Ph QA 33) 6.5 1-(isopropylamino)-3-(o-allylphenoxy)-2 propanol, HCl (H 56/28 = ptin®): 19· 4-(2-isopropylamino-1-hydroxyethyl) methane sulphonanilide, HCl (MJ 1999)· 24 2-(isopropylamino)-1 (p-nitrophenyl) ethanol, HCl (INPEA)· 50· dichloroisoprenaline (DCI)· no effect, α-adrenergic blocking compounds: chlorpromazine: > 50· phenoxybenzamine, HCl (dibenzyline®) no effect, phentolamine (regitin®). > 100. None of some commonly used antiarrhythmics: antazoline, HCl lidocaine, HCl, phenytoin, procainamide, HCl (pronestyl®) or quinidine sulphate had any effect. The most favourable therapeutic index (LD50/ED50) was found for (−)-propranolol ∼200. The method appears particularly suitable for evaluation of compounds with antagonistic action on cardiac β-receptors and in this respect appears to be highly sensitive.

Key-words: Mice – ventricular fibrillation – hydrocarbons halogenated – sympatholytics.

The antiarrhythmic effect of β-adrenergic receptor blocking compounds has in recent years been evaluated by several experimental test procedures. These may conveniently be divided into two aetiologically different groups. The first comprises methods in which adrenergic mechanisms are believed to play a minor role in the development of the arrhythmia. Among these are ventricular ectopic rhythms following experimental coronary ligation (HARRIS 1950) and ouabain infusion in dogs (LUCCHESI *et al* 1967) The other group comprises rhythm disturbances induced either by administration of adrenaline

or by substances or procedures, the actions of which are mediated through liberation of sympathomimetic amines. As a frequently used experimental model in the latter group fibrillation produced by an intravenous injection of adrenaline into cats and dogs may be mentioned in which the myocardium has been sensitized to adrenaline by an intratracheal injection of methylchloroform (1 1,1 trichloroethane) (SOMANI & LUM 1965).

During the search for convenient screening methods for evaluating antiarrhythmic properties we noticed that mice which had been heavily exposed to methylchloroform vapour died in cardiac fibrillation. A simple single step procedure based on this observation appeared suitable for screening purposes and the method was therefore elaborated further In order to characterize the sensitivity and specificity of the method a series of well established β-adrenergic blocking compounds was tested. The effect of a few α-adrenergic blocking agents and of some commonly used antiarrhythmic drugs was also investigated.

Material and Methods

Test drugs

The following compounds were used α-adrenergic blocking agents chlorpromazini chloridum NFN (klorpromazin Novo®) benzylytum NFN = phenoxybenzamine, HCl WHO (dibenzyline®), and phentolaminum NFN (regitin®) Antiarrhythmic drugs. Chinidini sulfas Ph. N lidocaini chloridum NFN phenytoinum NFN and procainamidi chloridum NFN (pronestyl®). β-adrenergic blocking compounds: 1-(isopropylamino)-3-(o-allylphenoxy)-2-propanol, HCl (H 56/28 = aptin®); dichloroisoprenaline, HCl (DCI) 2-(isopropylamino)-1-(p-nitrophenyl)ethanol, HCl (INPEA) 4-(2-isopropylamino-1 hydroxyethyl)methane sulphonanilide, HCl (MJ 1999)· 1-(isopropylamino)-3-(o-phenoxyphenoxy)- propanol, HCl (Ph QA 33) propranololi chloridum NFN = propranolol (inderal®) as well as the two optical isomers of the latter compound, methyl chloroform (1 1,1-trichloroethane, BDH), and reserpinum NFN All doses mentioned in the text refer to the form of the substances given in this paragraph except for phentolamine, which was given as th methanesulphonate. Th compounds were dissolved in saline at the required concentrations, except for phenoxybenzamine and reserpine which were dissolved in glacial acetic acid before dilution with saline.

Test for antifibrillatory effect.

Male NMRI mice weighing from 16–28 g, born SPF but kept under conventional conditions were used. All test compounds were administered intraperitoneally before exposure to methylchloroform. 30 min. after the drug administration the mice were transferred one by one to a 400 ml glass beaker containing cotton wool saturated with methylchloroform under a wire mesh at the bottom of the beaker This was covered with a watch glass The animals were now observed carefully and when respiratory arrest occurred they were *quickly removed from the beaker In order to* prevent the animal from recovering, th thoracic cavity was opened by an incision in the abdomen and the diaphragm without damaging the heart. The ECG was then recorded subcutaneously by bipolar leads according to SCHMITZEL (1933), the animals being properly *earthed between the electrodes.* The ECG was *recorded on* Mingo-

graph 12 (Elema) and in order to follow it continuously the output was fed into an oscilloscope (Tektronix, type 502A). The number of fibrillating mice in the pretreated groups (n = 10) was determined and the antifibrillatory effect expressed as th per cent decrease, as compared with a large control group of 320 mice. This was obtained by pooling smaller control groups of ten mice of which two groups at least were tested each day The effect was not quantitated in the singl mouse, i.e. if a test mouse showed even short runs of fibrillation the pretreatment was regarded as ineffective. The ED50 figures for the different compounds were compared with the LD50 in order to obtain a therapeutic index. The figures for LD50 intraperitoneally in table 1 and 2 are taken from a previous investigation (HERMANSEN 1969).

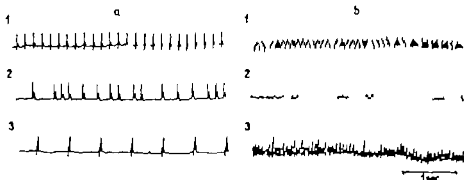


Fig. 1 Effect of different experimental procedures on the ECG in mice. At (1a) an ECG tracing from an untreated mouse anaesthetized with a barbiturate is shown. (2a) and (3a) are recorded at different times after cervical dislocation and bleeding in a mouse. Section (b) shows different patterns of methylchloroform arrhythmias (1) ventricular tachycardia, (2) fine fibrillation, and (3) coarse fibrillation.

Results

Effect of methylchloroform on cardiac rhythm.

Fig. 1 demonstrates the effect of methylchloroform on the ECG in the mouse. The upper left tracing (1a) shows a normal ECG of a barbiturate anaesthetized mouse with regularly occurring QRS complexes preceded by small but distinct P waves. The T wave follows immediately after the QRS complex. 2a and 3a show two strips of a terminal ECG after cervical dislocation and bleeding from the carotid arteries. The rhythm is slow and irregular but with distinct peaks and without any signs of ectopic activity such as fibrillation etc.

In section b of fig. 1 the different patterns of cardiac arrhythmias induced by methylchloroform are depicted. At (1) an ECG showing ventricular tachy cardia with a heart rate of 700–800 beats/min. is seen. This pattern of arrhythmias led either to fibrillation or to bradycardia and asystole. The most frequent arrhythmic pattern was that shown in (2) and (3) of section b. The

two tracings illustrate fibrillation of both low and high magnitude and are distinct from (1) by the absence of any regularity The latter picture (3) was the most frequently observed. When the ECG was followed on the scope the difference between (1) on the one hand and (2) and (3) on the other became particularly distinct. During the test period a total of 32 control groups of ten mice were investigated and 90 ± 2 / ($\bar{x}$ ± S E. M.) showed fibrillation of type (2) or (3). The effect of pretreatment with the test compounds was calculated from this gross mean.

Effect of β-adrenergic blocking compounds on methylchloroform fibrillations

During the initial studies it was noticed that very small doses of propranolol protected mice against the occurrence of fibrillation after respiratory arrest induced by methylchloroform. Fig. 2 shows the effect of the two β-blocking compounds, Ph QA 33 and propranolol. The upper tracing demonstrates the characteristic fibrillation of an untreated mouse exposed to methylchloroform, while section (a) and (b) show different stages of ECG s of mice pretreated with 3 mg/kg Ph QA 33 and 1 mg/kg propranolol, respectively The figure illustrates that the pretreated mice were protected against the fibrillatory action of methylchloroform.

Table 1 and 2 show a quantitative comparison of the antifibrillatory action of a series of β-adrenergic blocking compounds. The ED50 intraperitoneal values were obtained by a semilogarithmic plot of the percentage inhibition of graded doses calculated from the mean frequency of mice showing fibrillation in the large control group (n = 320) as mentioned previously The LD50 and the therapeutic index (LD50.ED50) are also listed for each compound.

DCI proved ineffective as a large dose of 50 mg/kg had only a marginal

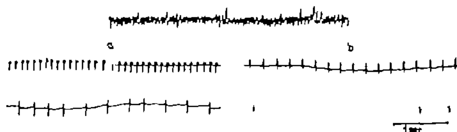


Fig. 2. Effect of pretreatment with Ph QA 33 and propranolol on the ECG in mice exposed to methylchloroform vapour The upper tracing shows fibrillation in a control mouse. Section (a) and (b) demonstrate th effect of pretreatment with 3 mg/kg Ph QA 33 (a) and 1 mg/kg propranolol (b) given intraperitoneally 30 min. before exposure to methylchloroform vapour

Table 1

Effect of different β-adrenergic blocking compounds on methylchloroform fibrillation in mice.

Compound	Dose mg/kg i. p.	% Inhibition	ED50 mg/kg i. p.	LD50 mg/kg i. p.	LD50/ ED50
DCI	50	11		110	
	100	tox.			
H 56/28	10	22	19	103	5.4
	20	33			
	30	77			
MJ 1999	10	11	24	790	32
	20	33			
	30	66			
INPEA	30	11	50	205	4.1
	50	44			
	100	tox.			
Ph QA 33	3	11	6.5	110	17
	5	22			
	10	77			

effect, while 100 mg/kg was toxic. INPEA and (+)-propranolol showed very weak activity and the effect demonstrated at 50 mg/kg should rather be regarded as unspecific because of the low therapeutic index of approximately 4 H 56/28 and MJ 1999 seemed to be about equiactive with ED50 values of 19 and 24 mg/kg intraperitoneally respectively However when the low toxicity of MJ 1999 is considered the therapeutic ratio of this compound is far greater than that of H 56/28. Ph QA 33 also proved to be quite specific in its antiarrhythmic action with an ED50 of 6.5 mg/kg and a therapeutic index of 17 Table 2 shows that the most effective compounds were by far the laevo rotatory isomer of propranolol and propranolol itself. The ED50 values of these substances were 0 6 and 1 7 mg/kg respectively and the therapeutic index of propranolol was 79 Because of shortage of supply of the (+)- and (−)-propranolol the accurate determination of the LD50 intraperitoneally has not been performed but it is of the same order as that of propranolol, i. e. 100–150 mg/kg intraperitoneally The protective effect of (+)-propranolol against methylchloroform induced fibrillations was only about one hundredth

Table 2

Effect of propranolol and its optical isomers on chloroform induced fibrillation in mice.

Compound	Dose mg/kg i. p.	% Inhibition	ED50 mg/kg i. p.	LD50 mg/kg i. p.	LD50/ED50
Propranolol ..	0 3	22			
	1	44			
	3	55	1.7	135	79
	10	77			
(+)-Propranolol	30	22			
	50	55	45		
	100	tox.			
(−)-Propranolol	0.3	33			
	1	66	0.6		
	3	88			

that of (−)-propranolol the same figure was obtained when their β-adrenergic blocking potencies were compared (Barret *et al.* 1968)

Protective action of α-adrenergic blocking compounds on methylchloroform induced fibrillations

In order to study the mechanism of methylchloroform induced arrhythmias further the effects of some α-adrenergic blocking compounds were also in-

Table 3

Effect of some -adrenergic blocking compounds on methylchloroform induced fibrillation in mice.

Compound	Dose mg/kg i. p.	% Inhibition	ED50 mg/kg i. p.
Chlorpromazine	5	22	> 50
	50	33	
Phenoxybenzamine	30	11	
	100	tox.	
Phentolamine	30	33	> 100
	100	44	

vestigated. Table 3 shows that pretreatment with chlorpromazine and phe noxybenzamine only had a negligible effect although the doses used were far above the effective α-blocking doses. Phentolamine showed some activity but only at the very high doses of 30 and 100 mg/kg.

Antifibrillatory effect of some commonly used antiarrhythmics

To investigate whether an antagonistic action against methylchloroform fibrillation could be produced by other groups of compounds than those with β-adrenergic blocking capacity a series of clinically useful agents were used in the study These agents are known to be active in different types of ex perimental arrhythmias. Table 4 shows the effect of five of the most commonly used antiarrhythmics and demonstrates clearly that antazoline, quinidine, lidocaine, phenytoin and procainamide were all ineffective. 100 mg/kg intraperitoneally was chosen as the maximal screening dose, which for all compounds except quinidine and procainamide represent a toxic dose level. The LD50's for these two compounds were determined to be about 210 and 325 mg/kg intraperitoneally respectively

Effect of interference with the adrenergic function on the development of arrhythmias.

The results described above indicate that methylchloroform fibrillation is brought about by mechanisms interfering with the normal adrenergic function.

Table 4

Effect of some commonly used antiarrhythmics on methylchloroform induced fibrillation in mice.

Compound	Dose mg/kg i. p.	% Inhibition	ED50 mg/kg i. p.
Antazoline	50 100	0 tox.	
Quinidine	50 100	0 11	> 100
Lidocaine	50 100	11 tox.	
Phenytoin	50 100	0 tox.	
Procainamide	100		> 100

To check this hypothesis three groups of 20 mice were exposed to methylchloroform after different pretreatments. The first group received 5 mg/kg reserpine intraperitoneally 16 hrs before the test, the next group was adrenalectomized 1 hr before exposure to methylchloroform and in the third group the two experimental procedures were combined. Although reserpinization causes an almost complete noradrenaline depletion in the mouse heart (MOORE 1964) the reserpine pretreatment alone did not afford any protection against the fibrillation. Adrenalectomy was also ineffective. Only the combined procedure had some effect as 50 / of the animals died without showing any fibrillation.

Discussion

Methylchloroform has previously been shown to sensitize the myocardium to the arrhythmogenic action of catecholamines and has therefore been given before the intravenous injection of adrenaline in order to obtain a consistent fibrillatory response in dogs (SOMANI *et al.* 1965) Methylchloroform administered alone has never been reported to cause arrhythmias, and the method described therefore provides a new means of inducing cardiac rhythm disturbances by a single step operation. A cardio-sensitizing effect of other chlorinated hydrocarbons has recently been reported by JOHNSON *et al.* (1968) They investigated 7 different compounds and found one of them (tetrachloromethane) capable of inducing cardiac arrhythmias in dogs without administration of adrenaline. Two other compounds, tri- and tetrachloroethylene, also gave rise to rhythm disturbances but only in combination with adrenaline.

As methylchloroform causes a non reversible respiratory arrest it would be of interest to investigate whether some other commonly used sacrificing procedures also induce cardiac arrhythmias. Fig. 1 shows that a widely used method which consists of cervical dislocation and bleeding only caused slowing of the heart rate, resulting in cardiac arrest without any signs of ectopic activity

When mice are sacrificed with chloroform, arrhythmias also develop as described by LAWSON (1967) However the incidence of fibrillating animals after chloroform exposure in our experience is too low to serve as a basis for a screening test. In the method described by LAWSON the absence of fibrillation was not taken as a criterion of effect, but the mice were regarded as protected when they showed a heart frequency of 200 or less.

As to the specificity of the method based on methylchloroform induced fibrillation, it appears from the results that only β-adrenergic blocking compounds are active in this test. The α-adrenergic blocking compounds, chlorpromazine and phentolamine, showed some activity at 30 and 100 mg/kg (table 3) i. e. far above those necessary to produce α-blockade (COURVOISIER

et al 1953 KARIM 1966) The lack of effect of some commonly used antiarrhythmic drugs is surprising in view of the fact that many of them cause a general cardiodepression and raise the fibrillation threshold in the same way as propranolol (PAPP *et al.* 1967) However it may be noted that rather high doses of this type of compound are needed in other antiarrhythmic tests despite the fact that a less severe arrhythmic pattern than fibrillation are employed (ventricular tachycardia etc.) The lack of effect of these antiarrhythmics against methylchloroform fibrillation therefore emphasizes the fact that the method described only provides a tool for the detection of substances with antagonistic action on cardiac β-receptors and cannot serve as a test for compounds effective against non-adrenergic mediated arrhythmias.

The adrenergic nature of methylchloroform induced fibrillation is documentated by the following facts. The order of potency of the compounds in table 1 and 2 corresponds well with that obtained in some other tests for β-adrenergic blocking activity Furthermore the antifibrillatory effect of (+)-propranolol was less than one per cent of the (−)-isomer which is the same ratio as that found for β-blocking potency (BARRET *et al* 1968). Finally the frequency of fibrillation was decreased by about 50 / in reserpinized and adrenalectomized animals. Reserpinization alone did not afford protection against methylchloroform although the myocardial storage sites are almost completely depleted of catecholamines. It is known that the adrenal amine stores in mice are more resistent to the depleting action of reserpine than in some other species (KRONEBERG *et al.* 1957) It is thus possible that the adrenal amines are responsible for the persistent action of methylchloroform in reserpinized mice.

The sensitivity of the method for detecting β-adrenergic blocking activity appears to be high as compared to some other tests. The ED50 of propranolol was found to be 1 7 mg/kg intraperitoneally which is of the same order as the antagonistic effect on the ouabain induced fibrillation in the guinea pig and the isoprenaline induced increase in myocardial force of contraction in cats (HERMANSEN 1968 & 1969) while LAWSON (1967) needed 35 mg/kg of propranolol intraperitoneally in order to get an effect against chloroform induced arrhythmias in mice.

The relative potency of the β-adrenergic blocking compounds listed in table 1 fits in reasonably well with previous findings. Thus the β-adrenergic blocking effect of Ph QA 33 has been previously found to be equal to propranolol in some tests and about one third that of propranolol in other tests (HERMANSEN 1968). The effect of H 56/28 was found to be only about 10 / of that of propranolol in spite of previously published observations that the β-blocking effect of this compound is equal to that of propranolol (ABLAD *et al* 1968). The postulated weak β-stimulant properties reported for both

H 56/28 and Ph QA 33 may be more dominant in this test and thus serve as an explanation for the discrepancy observed. In the same way the marked intrinsic activity of DCI may be responsible for the lack of effect of this compound. MJ 1999 was found to have the same activity as H 56/28 but with the favourable therapeutic index of 32. The poor effect of INPEA is in agreement with its weak β-blocking capacity (HERMANSEN 1969).

A new aspect in the development of β-adrenergic blocking compounds is the appearance of agents with selective blocking capacity (DUNLOP *et al.* 1967). The simple performance and apparent specificity on cardiac β receptors of the method described make it suitable for screening purposes and it may serve as a valuable tool in the further development of this field of work.

Acknowledgements

The author wishes to thank Mrs. I. Tofte Hyttel for her invaluable technical assistance. For a generous supply of samples of substances for the study I am indebted to the following firms

Eli Lilly & Co., Indianapolis, DCI Hässle AB Gothenburg, H 56/28 Selvi & Co Milano, INPEA Mead Johnson & Co Evansville, MJ 1999 and to Imperial Chemical Industries Ltd., Macclesfield for propranolol and its optical isomers.

REFERENCES

Ablad, B., M. Brogård & L. Ek. Pharmacological properties of H 56/28 – a β-adrenergic receptor antagonist. *Acta pharmacol. et toxicol* 1967 25 suppl. 2, 9–40

Barret, A. M & V A. Cullum. The biological properties of the optical isomers of propranolol and their effects on cardiac arrhythmias. *Brit. J Pharmacol* 1968 34 43–55

Courvoisier S., J Fournel, R. Ducrot, M. Kolsky & P Koetschet. Propriétés pharmacodynamiques du chlorhydrate de chloro-3 (diméthylamino-3'propyl)-10 phénothiazine (4 560 R. P). *Arch. int Pharmacodyn.* 1953 92, 305–361

Dunlop, D & R. G Shanks Selective blockade of adrenoceptive beta receptors in the heart. *Brit J Pharmacol* 1968, 32, 201 218

Harris, A. S. Delayed development of ventricular ectopic rhythms following experimental coronary occlusion *Circulation* 1950, 1 1318–1328

Hermansen, K. Some pharmacological properties of a new β-adrenergic blocking agent 1-(isopropylamino)-3-(o-phenoxyphenoxy)-2 propanol, HCl (Ph QA 33). *Acta pharmacol. et toxicol* 1968, 26 343–362.

Hermansen, K. Anti-arrhythmic, local anaesthetic, and adrenergic blocking activity of some β-receptor antagonists *Brit. J Pharmacol* 1969 35, 476–486.

Hermansen, K. Effect of different β-adrenergic receptor blocking agents on hexobarbital induced narcosis in mice. *Acta pharmacol et toxicol.* 1969 27 453–460.

Johnson, H. E. & S. P Shanor Electrocardiographic findings in dogs inhaling the vapors of diverse chlorinated hydrocarbons. *Toxicol Appl Pharmacol* 1968 12, 297

Karim, S. M. M.: The mechanism of the pressor action of noradrenaline in pithed cats. *Brit J Pharmacol* 1966, 27 17–32.

Kroneberg, G & H. J Schümann. Die Wirkung des Reserpins auf den Hormongehalt des Nebennierenmarks. *Arch. exp Path. Pharmakol.* 1957 231 349–360.

Lawson, J W Antiarrhythmic activity of some isoquinoline derivatives determined by rapid screening procedure in the mouse. *J Pharmacol exp Therap.* 1968, 160 22–31

Lucchesi, B. R., L. S. Whitsitt & J L. Stickney Antiarrhythmic effects of beta adrenergic blocking agents. *Ann. New York Acad Sci.* 1967 139 940–951.

Moore, K. E.: The role of endogenous norepinephrine in the toxicity of d-amphetamine in aggregated mice. *J Pharmacol exp Therap* 1964 144 45–51

Papp, G L. Szekeres & T Szmolenszky The effects of quinidine, ajmaline, papaverine and adrenergic beta-receptor inhibitors in experimental $BaCl_2$-arrhythmia developed for the quantitative assay of antiarrhythmic drugs. *Acta Physiol. Acad. Sci Hung* 1967 32, 365–375.

Schimmel, G. Das Elektrokardiogramm der kleinen Laboratoriumstiere. *Dissertation* 1933 München.

Somani, P & Bert K. B. Lum. The antiarrhythmic actions of beta adrenergic blocking agents. *J Pharmacol exp Therap* 1965 147 194–204

Acta pharmacol. et toxicol. 1970 28 28–38.

From the Department of Pharmacology University f Bergen, Norway

O Methylation of Simple Phenols in the Rat

By

Olav M. Bakke

(Received September 15 1969)

Abstract. Eleven simple mono- di- and trihydric phenols were given by stomach tube at a dose of 100 mg/kg body weight. The subsequent 48-hour urines were hydrolyzed, extracted and analyzed by thin-layer and gas chromatography and by infrared spectrophotometry The mean extent of direct *O*-methylation was 6.9 % with catechol, 6.0 % with 4-methylcatechol, 8.9 % with 4-ethylcatechol and 6.2 % with pyrogallol. Th preferred site of methylation was th middle hydroxyl group of pyrogallol and the *meta* hydroxyl group of 4-methylcatechol. Methoxyl groups were introduced *ortho* to the hydroxyl group of the monohydric phenols 4-ethylphenol, *p*-cresol and phenol, but this occurred t very small extent. No *O*-methylated metabolites were detected following the administration of resorcinol, hydroquinone, hydroxyquinol and phloroglucinol. There was no correlation between the extent of *O*-methylation of th simpl phenols and their potencies as convulsant agents. Formation of anisoles and *O*-methylation of more than one hydroxyl group were not observed with any of the compounds. The rats dosed with hydroxyquinol but not those given pyrogallol or phloroglucinol excreted small amounts of resorcinol. Metabolites produced by oxidation and unknown metabolites were also observed.

Key-words Methylation phenols – rats.

Our knowledge of the metabolism of phenolic compounds has been reviewed by WILLIAMS (1959) Phenols are metabolized mainly by conjugation to form glucuronides and ethereal sulphates which are excreted in the urine. Additional metabolic reactions are hydroxylation and *O*-methylation. *O*-Methylation of simple phenols has been shown to occur only with pyrogallol *in vivo* although the extent of this reaction is unknown (MASRI *et al.* 1962 SCHELINE 1966a).

Phenolic amines and acids possessing two adjacent phenolic groups are readily *O*-methylated by catechol-*O*-methyl transferase and this enzyme is

inhibited *in vitro* by catechol and pyrogallol (AXELROD 1966). The competitive inhibition of catechol-*O*-methyl transferase by these compounds is particularly interesting in view of the convulsant effect produced by simple phenols (DEICHMANN & WITHERUP 1944 ANGEL & ROGERS 1968) and the observation that their *O*-methylated derivatives are less toxic to laboratory animals (BINET 1895)

Previous investigations in this laboratory demonstrated the presence of catechols together with smaller amounts of their *O* methylated derivatives in the hydrolyzed urines of rats fed plant-containing diets and a purified casein sucrose diet added 10 / tyrosine (BAKKE 1969a & b) The present report gives qualitative and quantitative data on the formation of methyl ethers from various simple phenols following their administration to rats. The results are discussed in relation to the toxic properties of the simple phenols.

Methods

Compounds.

All test compounds and most of the methylated reference substances were obtained from commercial sources or were available in this laboratory (BAKKE & SCHELINE 1969a). Purity was checked by thin-layer and gas chromatography and the compounds were recrystallized or distilled if necessary 4-Ethylanisole, 2-*O*-methylpyrogallol and th methyl ethers of phloroglucinol, 4-methylcatechol, 4-ethylcatechol and hydroxyquinol were prepared by methylation of the phenols at room temperature in stirred acetone solutions to which equimolar quantities of potassium carbonat and dimethyl sulphate were added Th compounds were purified by distillation, if necessary with a spinning band column at reduced pressure (Nester & Faust MOF corp., Newark, Del.). The isomers of methylguaiacol and ethylguaiacol could not be separated but authentic samples of 4-methyl and 4-ethylguaiacol were available The yield of hydroxyquinol methyl ethers was poor but several minor peaks with shorter retention times than the parent phenol on column 5 (160°) were observed when the reaction mixture was analyzed. The various *O*-methylated hydroxyquinol derivatives are not included in tables 1 and 2.

Animal experiments

Male albino rats aged 5–6 months and weighing 350–370 g were used. They were maintained on commercial pellet diet (Felleskjøpet, Osl). Three days prior to dosing they were switched to a purified casein-sucrose diet (BAKKE 1969a). The animals were given free access to food and to water throughout the experiments.

Th test compounds dissolved in 1 ml of water unless otherwise indicated (table 4) were administered by stomach tube under light ether anesthesia at dose of 100 mg/kg body weight. By means f metabolic cages equipped with separators the individual urines were collected during the following 48 hours in containers which were kept below the freezing point. The rats were observed frequently following dosing and muscular twitching, convulsions and changes in consciousness were recorded.

Chromatography

The individual 48-hour urines were thawed, filtered and diluted to standard volume of 40 ml. Ten ml portions of th diluted urines were hydrolyzed with β-glucuronidase

(type H 1 containing sulphatase, Sigma Chemical Co St. Louis, Mo.) or in H_2SO_4, extracted and analyzed by thin-layer chromatography (table 1) and by gas chromatography (table 2) in several systems as described previously (BAKKE & SCHELINE 1969a & b BAKKE 1969a). With each test compound the hydrolysis method was chosen which in these previous studies gave the highest yield of the possible *O*-methylated metabolites. For the analysis of compounds which had not previously been investigated, both hydrolysis methods were used with separate urine samples. Extraction with sodium bicarbonate solution was omitted prior to thin-layer chromatography *p*-Methoxyphenol or 4-ethylphenol (0.3 mg in 1 ml of water) was added as internal standard before extraction for gas chromatographic analysis. The conversion of the test compounds to *O*-methylated urinary metabolites in 48 hours was calculated using internal standard compensation for losses.

Metabolit identification.

The methods of identification by gas chromatography on 3 columns and by thin-layer chromatography have been described in an earlier publication (BAKKE 1969a). In addi-

Table 1

Chromatography of some simpl phenols and their *O*-methylated derivatives on 0.5 mm thick layers of cellulose.)

Compounds	Rf Solvent 1	Rf Solvent 2	Colour with fast blue B salt followed by saturated $NaHCO_3$ solution
Pyrogallol	0.04	0.55	Pink-brown
Phloroglucinol	0.00	0 37	Blue-violet
Hydroxyquinol	0.00	0 48	Pink-grey
1-*O*-Methylpyrogallol	0 56	0 53	Grey
2-*O*-Methylpyrogallol	0.38	0.62	Violet
1,2 Di-*O*-methylpyrogallol	0 97	0 60	Red-orange
1,3-Di-*O*-methylpyrogallol ...	0.94	0.50	Blue-violet
Mono-*O*-methylphloroglucinol	0.11	–	Violet
Di-*O*-methylphloroglucinol	0.89	0.31	Violet
Catechol	0.30	0.62	Pink-grey
Resorcinol	0.10	0.56	Red-purple
Hydroquinone	0 09	0 62	Grey brown
m Methoxyphenol	0 87	0.51	Red-purpl
p-Methoxyphenol	0 84	0.54	Orange-brown
4-Methylcatechol	0.40	0 52	Grey-violet
4-Ethylcatechol	0 66	0 45	Pink-grey
Guaiacol	0.98	–	Orange
4-Methylguaiacol	0.98		Brown
4-Ethylguaiacol	0.98	–	Brown

) Sigmacell Type 19 (Sigma Chemical Co., St. Louis, Mo) was used with solvent 1 (benzene-glacial acetic acid-H_2O 6 7 3 upper layer) and MN 300 (Macherey Nagel & Co) was used with solvent 2 (20 % aqueous potassium chloride-glacial acetic acid, 100-1).

Table 2

Gas chromatography of some possibl O-methylated metabolites and reference simple phenols.*)

Compounds: Chemical names	Compounds: Trivial names	Column S 15 % w/w silicone rubber UC-W98, 120	Column C 1 % w/w Carbowax 20 M 130 → 185	Column T 2 % w/w tricresyl phosphate, 120
Methoxybenzene	Anisole	0.24	< 0.05	< 0.05
4-Methoxytoluene	4-Methylanisole	0.46	0.05	–
4-Hydroxytoluene	*p*-Cresol	0.58	0.72	1.00 (= 12 min.)
2 Methoxyphenol	Guaiacol	0.68	0.31	0.31
1 Ethyl-4-methoxybenzene	4-Ethylanisol	0.80	0.07	–
1,2-Dimethoxybenzene	Veratrole	0.86	0.19	0.28
1 Ethyl-4-hydroxybenzene	*p*-Ethylphenol	1.00 (= 4 min.)	1.00 (= 4.5 min.)	1.76
4-Hydroxy 3-methoxytoluene	4-Methylguaiacol	1.21	0.44	0.52
3-Hydroxy-4-methoxytoluene	5-Methylguaiacol	1.21	0.42	0.52
4-Methoxyphenol	*p*-Methoxyphenol, mono-*O*-methylhydroquinone	1.30	1.89	3.75
3-Methoxyphenol	*m*-Methoxyphenol, mono-*O*-methylresorcinol	1.36	2.07	4.70
3 4-Dimethoxytoluene	4-Methylveratrole	1.52	0.26	0.45
2,3-Dimethoxyphenol	1,2 Di-*O*-methylpyrogallol	1.58	0.78	1.00
1,2 Dihydroxy-3-methoxybenzene	1-*O*-Methylpyrogallol	1.76	2.32	2.85
1,3-Dihydroxy 2-methoxybenzene	2-*O*-Methylpyrogallol	1.76	3.10	–
4-Ethyl-2-methoxyphenol	4-Ethylguaiacol	1.94	0.61	0.83
2,6-Dimethoxyphenol	1,3-Di-*O*-methylpyrogallol	2.70	1.36	2.00
		Column S 160		
1,2,3-Trihydroxybenzene	Pyrogallol	1.00 (= 3 min.)	–	–
1,3,5-Trimethoxybenzene	Tri-*O*-methylphloroglucinol	1.14	–	–
3,5-Dimethoxyphenol	Di-*O* methylphloroglucinol	1.43	–	–
1,3-Dihydroxy-5-methoxybenzene	Mono-*O*-methylphloroglucinol	1.83	–	–

*) Relative retention times for the test compounds and the exact running conditions are given elsewhere (BAKKE & SCHELINE 1969a).

Table 3

O-Methylated metabolites in hydrolyzed 48-hour rat urines following intragastric administration of simple phenols (100 mg/kg body weight).

Test compounds (no. of rats)	Method of hydrolysis	Method of analysis	*O*-Methylated metabolites. Mean % conversion with range in brackets
Pyrogallol	Enzymic	GLC and TLC*	2-*O*-Methylpyrogallol 6.2 (4.5-8.1)
(5)	Acid	GLC and TLC	2-*O*-Methylpyrogallol, smaller amounts than with enzymic hydrolysis. 1-*O*-Methylpyrogallol in traces? (Col. C only)
Hydroxyquinol	Enzymic	GLC and TLC	None
(5)	Acid	GLC and TLC	None
Phloroglucinol	Enzymic	GLC and TLC	None
(5)	Acid	GLC and TLC	None
Catechol (5)	Enzymic	GLC and IR	Guaiacol 6.9 (5.6-7.4) Veratrole not detected
4-Methylcatechol (5)	Enzymic	GLC and IR	4-Methylguaiacol and 5 Methylguaiacol 6.0 (4.0-8.1) 4-Methylveratrole not detected
4-Ethylcatechol (5)	Enzymic	GLC and IR	Ethylguaiacols 8.9 (6.6-11.7) 4-Ethylveratrole not detected
Resorcinol (5)	Enzymic	GLC	None
Hydroquinone (5)	Enzymic	GLC	None
Phenol (4)	Enzymic	GLC	Guaiacol 0.1 (all 0.1) Anisol not detected
p-Cresol (5)	Enzymic	GLC	4-Methylguaiacol and 5 Methylguaiacol 0.2 (0.1-0.3) 4-Methylanisole not detected
4-Ethylphenol (5)	Enzymic	GLC	Ethylguaiacols < 0.1 (all < 0.1) 4-Ethylanisole not detected

Symbols. GLC = gas chromatography TLC = thin-layer chromatography IR = infrared spectrophotometry

tion, some new metabolites were identified by infrared spectrophotometry By means of a splitter arrangement the effluent gas flow was diverted from the gas chromatographic column to a heated line Silver chloride cells with 0.1 mm path length (Research and Industrial Instruments Co., London) were positioned at th exit of the heated line and the eluted metabolites were condensed by immersion of the cells in mixture of ethanol and solid carbon dioxide. The metabolites were carried down to the bottom of the cells by repeatedly washing the cell walls with 10 μl of diethyl ether which was subse-

quantly evaporated. After the addition of 2 µl of carbon tetrachloride the spectra were obtained with a Hilger H900 Infrascan recording spectrophotometer equipped with a beam condenser (model C-621 Research and Industrial Instruments Co., London). Spectra of reference compounds dissolved in carbon tetrachloride were also obtained with silver chloride cells.

Results

The urinary methyl ethers of the administered phenols are listed in table 3 Infrared spectra were obtained with the metabolites of catechol, 4-methylcatechol and 4-ethylcatechol. Reference guaiacol yielded a spectrum identical to that obtained with the catechol metabolite collected from column C. Gas chromatographic analysis showed that 4-methylcatechol and p-cresol were metabolized to both 4-methyl and 5-methylguaiacol and the ratio of the amounts produced were approximately 2 1 in favour of the former isomer These isomers were trapped collectively from column C and their infrared absorption maxima were identical to those of 4-methylguaiacol although some of the bands were broader as with the synthetic mixture of 4-methyl and 5-methylguaiacol.

Analysis of the reaction mixture in the chemical methylation experiment with 4-ethylcatechol suggested that 4-ethyl- and 5-ethylguaiacol elute together from all of the columns used. After the ingestion of 4-ethylcatechol and 4-ethylphenol peaks with the retention characteristics of these ethylguaiacols appeared on the chromatograms. The infrared spectrum of this metabolite collected from column S was compared to the spectra obtained with reference 4-ethylguaiacol and with a mixture of 4-ethyl- and 5-ethylguaiacol produced synthetically Although the trapped compound showed the characteristic absorption of an ethylguaiacol it could not be determined which of the isomers is the major metabolite.

Owing to the coincidence of the pyrogallol monomethyl ethers on column S the possible trace of 1-O-methylpyrogallol in acid-hydrolyzed urines follow ing pyrogallol administration could not be confirmed on this column in the presence of large amounts of its isomer and it was not detected by the less sensitive thin layer chromatographic method. Methylation of more than one hydroxyl group was not observed with any of the compounds.

In animals given p-cresol and 4-ethylphenol thin-layer and gas chromatography failed to demonstrate the urinary excretion of their respective hy droxylated derivatives, 4-methylcatechol and 4-ethylcatechol. Phenol was hydroxylated to hydroquinone and to a small extent to catechol. None of the monohydric phenols were converted to their corresponding anisoles.

Extracts of the enzyme-hydrolyzed urines of rats given catechol, 4-methylcatechol and 4-ethylcatechol yielded small peaks on column S (120°) with

relative retention times of 3 60 6.0 and 9 8, respectively These unknown metabolites eluted in the same order on column C with the first compound coming immediately after *p*-methoxyphenol and the other compounds 2 and 3 minutes after this internal standard, respectively Also in the experiments with 4-ethylphenol some unknown metabolites gave peaks on the chromatograms and 4-hydroxyphenylacetic acid was detected by thin layer chromatography of the urine extracts. Prominent spots corresponding to 4-hydroxybenzoic acid were observed on the thin layer chromatograms following the administration of *p*-cresol.

The methyl ethers of hydroxyquinol were not available as reference compounds and their general gas and thin-layer chromatographic behaviour was indicated using the reaction mixture of dimethyl sulphate-treated hydroxy quinol. None of these compounds were detected on chromatograms of the hydrolyzed urines of hydroxyquinol-treated animals.

Thin-layer chromatography of extracts from enzyme-hydrolyzed urines showed that a very small amount of resorcinol was excreted following dosing with hydroxyquinol while only 2 of the acid-hydrolyzed urines yielded this

Table 4

Motor disturbances following intragastric administration of simple phenols (100 mg/kg body weight) to groups of 5 rats.

Test compounds	Vehicl	Symptoms
Pyrogallol	1 ml of water	None in 5/5
Hydroxyquinol	1 ml of water	None in 5/5
Phloroglucinol	1 ml of propylene glycol water 1 1	None in 5/5
Catechol	1 ml of water	Severe convulsions in 5/5 coma in 3/5
4-Methylcatechol	1 ml of water	Moderate convulsions in 1/5 moderate muscular twitching in 4/5
4-Ethylcatechol	1 ml of water	Slight muscular twitching in 1/5 none in 4/5
Resorcinol	1 ml of water	Severe convulsions in 5/5 coma in 2/5
Hydroquinone	1 ml of water	Severe convulsions and coma in 1/5 moderate convulsions in 2/5 moderate muscular twitching in 2/5
Phenol	1 ml of water	Severe convulsions and coma I 2/5 followed by death in 1/5 moderate muscular twitching in 3/5
p-Cresol	2 ml of water	Slight muscular twitching in 5/5
4-Ethylphenol	1 ml of propylene glycol-water 1 1	None in 5/5

metabolite. Resorcinol was not detected in the urine of rats given pyrogallol or phloroglucinol.

The acute motor disturbances induced by the simple phenols are summarized in table 4. Muscular twitching and convulsions started 4–10 minutes after dosing with phenol, catechol, 4-methylcatechol, resorcinol and hydroquinone and lasted for 10–45 minutes. With *p*-cresol and 4-ethylcatechol muscular twitching but no convulsions were observed. In the rats receiving 4-ethylphenol, pyrogallol, phloroglucinol and hydroxyquinol and in control animals dosed with water under ether anaesthesia no excitation phenomena were observed.

Discussion

The formation of 2-*O*-methylpyrogallol from pyrogallol *in vivo* has been demonstrated by MASRI *et al.* (1962) and by SCHELINE (1966a). This metabolic reaction was reproduced *in vitro* with catechol-*O*-methyl transferase from rat liver (MASRI *et al.* 1964). The present results confirm that 2-*O*-methylpyrogallol is produced from pyrogallol *in vivo* and demonstrate that this conversion occurs to a considerable extent. 1,2-Di-*O*-methylpyrogallol which ARCHER *et al.* (1960) claimed to be the main *O*-methylated metabolite *in vitro* was not detected.

SCHELINE (1966b) demonstrated the dehydroxylation of pyrogallol and hydroxyquinol to yield resorcinol in incubates of rat caecal contents but found that phloroglucinol was not so affected. Traces of resorcinol have also been detected in the hydrolyzed urine of rats dosed with pyrogallol (SCHELINE 1966a). While the latter *in vivo* finding could not be confirmed by the present study the dehydroxylation of hydroxyquinol was shown to take place also in the intact animal.

The failure of resorcinol and hydroquinone to yield methylated metabolites while catechol, 4-methylcatechol and 4-ethylcatechol yielded considerable quantities of such derivatives demonstrates that the 2 adjacent hydroxyl groups are essential for direct *O*-methylation *in vivo*. Hydroxyquinol is the only compound with this configuration which was not shown to produce a methyl ether in the present study. This compound, however, is very unstable and little is known about its fate in the animal body except that it forms a glucuronide (see WILLIAMS 1959).

GARTON & WILLIAMS (1948) studied the urinary metabolites of catechol following its oral administration to rabbits and found that approximately 90 % of the dose appeared in the urine as conjugated and free catechol. A trace of hydroxyquinol was also demonstrated in these urines. The present study demonstrates that guaiacol is an additional metabolite of catechol and

the quantities produced by the rats correspond fairly well to the fraction of ingested catechol which has not been accounted for in rabbit urine. This finding confirms an earlier suggestion that guaiacol which occurs in the urine of rats fed plant-containing diets may arise from catechol (BAKKE 1969a). Also, 4-methylguaiacol which was detected in these urines and in the urine of rats fed a 10 / tyrosine diet is possibly produced by *O*-methylation of 4-methylcatechol derived from plant constituents and from tyrosine (BAKKE (1969b) The predominance of 4-methylguaiacol over its 5 methyl isomer in the present experiments is consistent with the observations of earlier investigators with catecholamines and with most of the short chain phenolic acids studied as these are methylated mainly at the *meta* hydroxyl group by catechol-*O*-methyl transferase *in vitro* and in intact animals (DEEDS *et al* 1957 MASRI *et al* 1962 MASRI *et al.* 1964 AXELROD 1966).

Aromatic hydroxylation of phenol in the rabbit to yield hydroquinone, catechol and a trace of hydroxyquinol was demonstrated by GARTON & WILLIAMS (1949) BRAY *et al* (1950) found a trace of conjugated 4-methylcatechol in the urine of rabbits given *p*-cresol by stomach tube (100–200 mg/kg body weight). The metabolism of 4-ethylphenol has not been investigated previously but as with *p*-cresol its *ortho* dihydroxy derivative was not detected in the urine in the present experiments with rats. However the excretion of small amounts of guaiacols following the administration of these compounds suggests the formation of catechol derivatives and is consistent with the findings of DALY *et al.* (1965) who presented evidence for microsomal hydroxylation of phenols and subsequent *O* methylation by catechol-*O*-methyl transferase *in vitro*

The relative retention times of the unknown metabolites of catechol 4-methylcatechol and 4-ethylcatechol suggest analogous metabolism of these compounds. The peak observed on columns C and S following catechol administration was not present when the hydroxylated metabolite hydroxy quinol was given. Therefore, in contrast to the findings with the monohydric phenols, there was no evidence that hydroxylation of catechols is followed by *O*-methylation. With the alkyl substituted phenols some of the unknown peaks might be accounted for by side chain oxidation to aromatic alcohols which are extracted together with the simple phenols. Side chain oxidation to phenolic acids was demonstrated with *p*-cresol and 4-ethylphenol.

The convulsant effects of some of the test compounds included in the present study have been described by other workers (BINET 1895 DEICHMANN & WITHERUP 1944 ANGEL & ROGERS 1968) The doses which are required to elicit convulsions in laboratory animals are different for most of the compounds studied and the data in table 4 are given only to indicate the relative toxicities of the test compounds at the dose level used in the present metabolic study To dissolve the standard dose of *p*-cresol 2 ml of water was

required. However DEICHMANN & WITHERUP (1944) have demonstrated that the concentration of aqueous solutions given orally does not affect the acute systemic toxicity of phenol in rats. 4-Ethylphenol and phloroglucinol were dissolved in equal parts of water and propylene glycol, a solvent which might influence the absorption rate and therefore the acute toxic manifestations. With the remaining test compounds which were dissolved in water only there is a tendency for the convulsant potencies to decrease with alkyl substitution and with the chain length of the substituents. Also the introduction of more than two phenolic groups in the molecule is accompanied by reduced potency

As review recently by ANGEL *et al.* (1969) various hypotheses have been put forward to explain the convulsant actions of catechol and pyrogallol The possibility of decreased inactivation of cerebral monoamines due to inhibition of catechol-O-methyl transferase by these phenols has been considered. ROGERS *et al.* (1968) have shown that catechol and pyrogallol begin to enter the brain within the first minute after their intraperitoneal injection in mice and that the time course of catechol penetration into the brain follows closely the time course of the convulsive activity However they found no change in the concentration of monoamines in the brain following the administration of these compounds. On the other hand, IZQUIERDO *et al* (1964) reported that intraperitoneal injection of pyrogallol increased the concentration of catecholamines in some areas of the brain in rats and mice. The present results suggest no correlation between the convulsant effect of the simple phenols and their ability to undergo O-methylation *in vivo*

BINET (1895) studied the acute toxicity of several simple phenols and some O-methylated derivatives in various laboratory animals including the rat. These derivatives were found to be much less toxic than the corresponding phenols. Therefore, as with the catecholamines which exhibit a decrease in biological activity as a result of O methylation (AXELROD 1966) this metabolic pathway is to be regarded as a detoxication mechanism with the simple phenols.

Acknowledgements

The technical assistance of Mrs. Eli Tepstad and Mr Olav Fjellbirkeland is greatly appreciated. Some of the instruments used in the present study have been purchased with grants from Norges Almenvitenskapelige Forskningsråd, Norsk Medisinaldepot and from Det Videnskapelige Forskningsfond av 1919 Oslo.

REFERENCES

Angel, A & K. J Rogers: Convulsant action of polyphenols. *Nature (Lond.)* 1968, 217 84–85

Angel, A., R. N Lemon, K. J Rogers & P Banks The effect of polyhydroxyphenols on brain ATP in the mouse. *Exp Brain Res* 1969 7 250–257

Archer S A. Arnold, R. K. Kullnig & D W Wylie: The enzymic methylation of pyrogallol. *Arch. Biochem Biophys* 1960, 87 153–154

Axelrod, J.. Methylation reactions in the formation and metabolism of catecholamines and other biogenic amines. *Pharmacol Rev* 1966 18, 95–113

Bakke, O M.. Urinary simple phenols in rats fed purified and nonpurified diets. *J Nutr* 1969a, 98, 209–216.

Bakke, O M.. Urinary simple phenols in rats fed diets containing different amounts of casein and 10 % tyrosine. *J N tr* 1969b, 98, 217–221.

Bakke, O M. & R. R. Scheline: Analysis of simple phenols of interest in metabolism. I. Gas chromatographic separation and quantitative determination. *Anal Biochem.* 1969a, 27 439–450.

Bakke, O M. & R. R. Scheline: Analysis of simple phenols of interest in metabolism. II. Conjugate hydrolysis and extraction methods. *Anal. Biochem.* 1969b, 27 451–462.

Binet, P Toxicologie comparée des phénols. *Rev méd. Suisse rom* 1895 15 617–659

Bray H. G W V Thorpe & K. White: Metabolism of derivatives of toluene. 4 Cresols. *Biochem. J* 1950, 46 275–278.

Daly J J K. Inscoe & J Axelrod. The formation of O-methylated catechols by microsomal hydroxylation of phenols and subsequent enzymatic catechol O-methylation Substrate specificity *J med. Chem.* 1965 8 153–157

DeEds, F A. N Booth & F T Jones: Methylation and dehydroxylation of phenolic compounds by rats and rabbits. *J biol. Chem* 1957 225, 615–621

Deichmann, W B & S. Witherup Phen l studies VI. Th acute and comparative toxicity of phenol and *o- m-* and *p*-cresols for experimental animals. *J Pharmacol. exp Ther* 1944, 80 233– 40

Garton, G. A. & R. T Williams. Studies in detoxication. 17 The fate of catechol in the rabbit and the characterization of catechol monoglucuronide. *Biochem. J* 1948, 43, 206–211

Garton, G A. & R. T Williams. Studies in detoxication. 26. Th fates of phenol, phenylsulphuric acid and phenylglucuronide in the rabbit, in relation to the metabolism of benzene. *Biochem. J* 1949 45, 158–163

Izquierdo, J A., L J Jofre & M. A. Dezza. Effect of pyrogallol on the catecholamine contents of cortex diencephalon, mesencephalon, rhombencephalon and cerebellum of mouse and rat. *Med exp.* 1964 10 45–55

Masri, M S., A. N Booth & F DeEds: O-Methylation *in vitro* of dihydroxy and trihydroxy phenolic compounds by liver slices. *Biochim. Biophys Acta* 1962, 65, 495–505

Masri, M S., D J Robbins, O H. Emerson & F DeEds. Selective *para-* or *meta-O-*methylation with catechol O-methyl transferase from rat liver *Nature (Lond.)* 1964, 202, 878–879

Rogers, K J A. Angel & L. Butterfield The penetration of catechol and pyrogallol into mouse brain and the effect on cerebral monoamine levels. *J Pharm. Pharmacol* 1968. 20 727 729

Scheline, R. R. Th decarboxylation of some phenolic acids by the rat. *Acta pharmacol. et toxicol* 1966a, 24 275–285

Scheline, R. R. Decarboxylation and demethylation of some phenolic benzoic acid derivatives by rat caecal contents. *J Pharm. Pharmacol* 1966b 18 664–669

Williams, R. T *Detoxication Mechanisms*, 2nd ed. Chapman and H ll, London 1959 pp. 278–317

Acta pharmacol. et toxicol. 1970 **28**, 39–48

From the Department of Pharmacology University of Göteborg,
and the Department of Histology Karolinska Institutet,
Stockholm 60, Sweden

Protection of the Neostriatal Dopamine Stores against Reserpine by Local Treatment with Metatyramine

By

Nils-Erik Andén, Sherrel G. Butcher and Kjell Fuxe

(Received July 24, 1969)

Abstract Metatyramine (15 mg in 5 hrs) was infused unilaterally into the corpus striatum of rats while the same volume of saline was given contralaterally. Reserpine given simultaneously (2.5 mg/kg intraperitoneally) caused a significantly smaller reduction of dopamine on the metatyramine-treated side after 25 hrs. Th noradrenaline was protected to a smaller extent. At the same time there was a turning of the rats to the opposite side. Four hours after a second injection of reserpine (5 mg/kg intraperitoneally), negligible amounts of dopamine were found on both sides and the asymmetries had disappeared. Since the asymmetries were more marked 24 hrs later than during th metatyramin infusion, the protection of th dopamine on one side was probably responsible for the asymmetries.

Key-words: Metatyramine – reserpine – dopamine stores – neostriatum.

Previous studies on mice have shown that repeated systemic injections of metatyrosine just before and after reserpine injection can partially protect the dopamine (DA) and noradrenaline (NA) stores in the brain from depletion by reserpine (Carlsson & Lindqvist 1967). This protection is accompanied by a concomitant antagonism of both the reduction in locomotion and the decreased reactivity to stimuli usually observed after reserpine treatment. In this respect the protection of brain DA appears to be of great importance. The protection is probably produced by metatyramine and β-hydroxy-metatyramine which are formed from metatyrosine in the DA and NA neurons. These amines may compete with reserpine at the amine uptake sites of the catecholamine granules. In view of these previous findings we have studied the effects of direct intrastriatal application of metatyramine on the reserpine induced depletion of neostriatal DA stores and on the reserpine-induced changes in the neostriatal DA transmission.

Material and Methods

Sprague-Dawley rats weighing between 250 and 350 g were used. Drugs were introduced into th neostriatum by means of a double cannula assembly similar to that described by GROSSMAN (1964). The assembly consisted of a guide and an injection cannula both made of stainless steel. Under pentobarbital anesthesia the guide cannulas (inner diameter 0.89 mm, outer diameter 1.25 mm) were permanently implanted bilaterally using a stereotaxic procedure. Acrylic cement was used to secure the cannulas in place. Th rats were allowed to recover from surgery for at least one week before the start of experimentation. The injection cannulas were inserted bilaterally into the guide tubes of unanesthetized rats. Drugs were administered into the corpus striatum through the injection cannulas (inner diameter 0.41 mm, outer diameter 0.71 mm). When in place the tip of the injection cannulas had the following coordinates (KÖNIG & KLIPPEL 1963) anterior + 7.9 mm, lateral ± 2.7 mm, vertical + 4.0 mm.

A solution of metatyramine HCl (50 mg/ml redistilled water) was infused into th right corpus striatum for a period of 5 hours at a rat of 1 µl/min. As a control an equal volume of 0.9 per cent saline was infused simultaneously into the corpus striatum on the opposite side. Tw hours after th beginning of the infusion, reserpine was injected (2.5 mg/kg, intraperitoneally) The infusion was then continued for 3 hours. The animals were sacrificed by decapitation 4 hours after the reserpine injection. In 3 separate experiments on 8 rats, a second dose of reserpine (5 mg/kg intraperitoneally) was given 24 hours after the first one and the rats were sacrificed 4 hours later

The total volume infused into the corpus striatum over the period of 5 hours was about 300 µl per side. Sometimes the amount was probably somewhat smaller i.e. when one or both cannulas were removed by the animals themselves or by the investigators for short periods of time. Moreover after the infusion had been in progress for several hours small amounts of fluid came out of the top of the guide cannula in many experiments.

In the biochemical experiments, the right and left forebrain was analyzed spectrofluorometrically for DA and NA after cation exchange chromatography and oxidation (BERTLER, CARLSSON & ROSENGREN 1958 CARLSSON & WALDECK 1958 CARLSSON & LINDQVIST 1962).

In separate experiments the metatyramin and β-hydroxy-metatyramine of the pooled brain halves from 2 or 3 rats were purified and separated by cation exchange chromatography nd determined colorimetrically after addition of Gibbs reagent (MITOMA, POSNER, BOGDANSKI & UDENFRIEND 1957) as described for α-methyl-metatyramine and metaraminol (ANDÉN 1964). Untreated rat brains served as blank controls and showed colour The recoveries of 1 µg metatyramine and 1 µg β-hydroxy-metatyramine added to a brain extract were 100 and 116 per cent, respectively

Histochemical analyses of DA, NA and 5-hydroxytryptamin in different regions of the rat brain were also made. After decapitation of the rats the telencephalon, diencephalon and mesencephalon were rapidly dissected out, freeze dried and allowed t react with formaldehyde gas as previously described in detail (FALCK, HILLARP THIEME & TORP 1962, DAHLSTRÖM & FUXE 1964 CORRODI & JONSSON 1967). The fluorescence microscopy was performed on coded preparations independently of the biochemical results.

The statistical significance between the values obtained from the two brai halves was calculated by the t-test for the difference between the means of paired samples (SNEDECOR & COCHRAN 1967) The differences were considered not significant at P values higher than 0.05

Results

Biochemistry

In the reserpine treated rats infused with metatyramine into the right corpus striatum, the DA remaining in the right forebrain was significantly higher than in the left forebrain (table 1) The NA was also significantly higher on the right than on the left side (table 1). After unilateral infusion of saline for 5 hours into the corpus striatum of non-reserpine treated rats, the levels of DA and NA in the forebrain on the treated side were 0 74 ± 0 107 (n = 2) and 0 49 ± 0 042 (n = 2) μg/g, respectively The differences between the two sides in the rats treated with metatyramine and reserpine were 18 and 8 per cent of the normal values for DA and NA, respectively (table 1)

When a second dose of reserpine (5 mg/kg intraperitoneally) was given to protected rats 24 hours after the first dose, and the rats killed 4 hrs later the DA and NA values were reduced to approximately the same level on both sides (DA:right = 0 071 μg/g, left = 0 102 μg/g NA:right = 0 032 μg/g, left = 0 031 μg/g; mean of 3 experiments).

The amounts of metatyramine found in the rat brain after treatment with metatyramine plus reserpine were variable (table 2) There was, however always a higher concentration on the right side. The content of β-hydroxy-metatyramine was negligible on both sides in all experiments except one.

Histochemistry

After infusion of metatyramine into the right corpus striatum and injection of reserpine (13 rats), there was a weak to moderate diffuse green fluorescence in the right caudate nucleus-putamen (neostriatum) However the left neostriatum was virtually devoid of specific fluorescence (fig. 1a and b). The fluorescence of the DA nerve terminals in the neostriatum of untreated rats was intense (fig. 1c) The fluorescence observed in the right neostriatum in the present experiments was therefore weaker than normal The fluorescent DA nerve terminals were usually not seen in the entire right neostriatum but mainly in the medial-dorsal part. In the part of the right neostriatum close to the tip of the cannula there was no fluorescence. This lack of fluorescence may be due to the remaining metatyramine in the nerve terminals around the tip. Intraneuronally located metatyramine could not be observed in the ordinary fluorescence microscope since the emission peak of the blue fluorescence emitted from the 6-hydroxy-3 4-dihydroisoquinoline formed is around 415 mμ (CORRODI & JONSSON 1966 JONSSON & RITZÉN 1966) which can not be observed with the secondary filter used (strong absorption below 480 mμ)

The DA nerve terminals in the tuberculum olfactorium, nucleus accumbens and nucleus interstitialis striae terminalis showed practically no fluorescence on the right or on the left side. A few weakly fluorescent NA nerve terminals

Table 1

Levels ($\mu g/g$) of dopamine and noradrenaline in the forebrain halves of rats. The rats were infused with metatyramine HCl (15 mg/300 μl) into the right corpus striatum 26–21 hours before sacrifice. They were injected with reserpine (2.5 mg/kg intraperitoneally) 24 hours before sacrifice. The figures in parentheses denote the number of experiments. The figures in brackets are expressed in per cent of the normal value.

Amine	Right brain half M ± S. E. M.	Left brain half M ± S. E. M	Difference d ± S. E. (diff.)
Dopamine	0.225 ± 0.0399 (11) [32 %]	0.103 ± 0.0165 (11) [14 %]	0.122 ± 0.0426 $P < 0.025$
Noradrenaline	0.118 ± 0.0317 (11) [24 %]	0.080 ± 0.0245 (11) [16 %]	0.037 ± 0.0090 $P < 0.005$

Table 2

Levels (μg/g) of metatyramine and β-hydroxy metatyramine in th two brain halves of rats. The rats were infused with metatyramine HCl (15 mg/300 μl) into the right corpus striatum 26–21 hours before sacrifice. They were injected with reserpine (2.5 mg/kg intraperitoneally) 24 hours before sacrifice. Each row of individual values is from the same experiment for both metatyramine and β-hydroxy-metatyramine.

Amine	Right brain half	Left brain half	Difference
Metatyramine	0.653	0.201	0.452
	0.076	0.031	0.045
	1.815	1.544	0.271
	0.640	0.100	0.540
	0.394	0.138	0.256
	0.057	0.051	0.006
	0.606 ± 0.264 [M ± S. E. M.]	0.344 ± 0.241 [M ± S. E. M.]	0.262 ± 0.087 [d ± S. E. (diff.)] (P < 0.05)
β-Hydroxy-metatyramine	− 0.094	− 0.048	− 0.046
	− 0.061	− 0.126	0.065
	0.203	0.137	0.066
	− 0.077	− 0.010	− 0.067
	0.069	0.056	0.013
	− 0.275	− 0.289	0.014
	− 0.039 ± 0.066 [M ± S. E. M.]	− 0.047 ± 0.061 [M ± S. E. M.]	0.0075 ± 0.0225 [d ± S. E. (diff.)] (P > 0.1)







Fig. 1a (top). The left saline-treated caudate nucleus of a rat 4 hours after reserpine treatment (2.5 mg/kg intraperitoneally). The right caudate nucleus of the same rat is shown in fig. 1b Practically no fluorescence is seen in the left caudate nucleus. v = lateral ventricle. Magnification 120 ×

Fig. 1b (middl). The right caudate nucleus of a rat 24 hours after reserpine (2.5 mg/kg intraperitoneally) treatment and local infusion of metatyramine (about 15 mg) into the right corpus striatum. For further details, see text. I the part of the caudate nucleus close to the lateral ventricle ( ) there is diffuse green fluorescence of moderate intensity Magnification 120 ×

Fig. 1c (bottom). The right caudate nucleus of a normal rat. Ther is diffuse green fluorescence of strong intensity Magnification 120 ×

remained and they were found in about equal numbers on both sides of the brain.

Five rats were sacrificed immediately after the metatyramine infusion and studied histochemically. A green-yellowish fluorescence of very strong intensity was observed in the medial and dorsal but not in the ventral and lateral part of the right neostriatum (fig. 2). The major part of the green-yellowish fluorescence on the treated side was located in the corpus striatum. On the saline-treated side, such fluorescence was not observed. The yellow fluorescence was in all likelihood due to the presence of high concentrations of metatyramine mainly located extraneuronally. Thus, under these conditions, when not present intraneuronally, the fluorophor metahydroxy-3,4-dihydroisoquinoline formed from metatyramine is converted into its tautomeric quinoidal form (emission peak around 510 mμ) and exhibits a green-yellowish fluorescence in the ordinary fluorescence microscope with the secondary filter used (CORRODI & JONSSON 1966, JONSSON 1966, JONSSON & RITZÉN 1966). The cannula tracts were easily seen under the microscope since they were surrounded by cells with an orange fluorescence of strong intensity. In an area 200–400 mμ in diameter immediately around the cannula, necrosis of the brain tissue was seen.

Function.

The infusion of metatyramine into the right corpus striatum caused the rats to turn or rotate to the left side. This asymmetry was usually noted after



Fig. 2. The right caudate nucleus of a rat immediately after local infusion of metatyramin (about 15 mg). For further details, see text. The whole medio-dorsal part of the caudate nucleus is seen to contain a diffuse green-yellowish fluorescence of very strong intensity. The yellow fluorescence probably reflects the presence of high concentration of metatyramine which is mainly located extraneuronally. S = septal area. Magnification 80 ×

approximately 90 minutes. The asymmetry became more pronounced after the reserpine injection and remained throughout the 24 hour period. In all experiments the rats remained asymmetrical up to the time of sacrifice with the head and tail turned to the left and with the hindleg abduced on the right side and adducted on the left side. There seemed to be a positive correlation between the degree of asymmetry and the difference in DA between the two sides.

When the second dose of reserpine (5 mg/kg intraperitoneally) was given 24 hours after the first dose the asymmetry was eliminated in all the 8 rats investigated.

Discussion

The present study has shown both biochemically and histochemically that infusion of metatyramine into the neostriatum can partially protect the neostriatal DA stores from depletion by reserpine. That there actually was a protection of the DA stores on the right side was demonstrated by the fact that a second dose of reserpine reduced the remaining DA at least as much on the right as on the left side. The latter findings also indicated that the protected DA was localized in the amine storage granules of the nerve terminals.

There was also a partial protection of the NA stores on the side treated with metatyramine. This is not unexpected since the volume of metatyramine used was rather large and must also have reached regions outside the corpus striatum. The NA was, however protected to a smaller extent than the DA on the right side. On the left side the NA and the DA were depleted to about the same extent.

All the rats showed a turning from the side with the highest DA value and the asymmetry was most pronounced in the animals with the largest difference in DA between the two sides. This effect is in all likelihood due to a better neurotransmission in the neostriatum on the treated side since a similar asymmetry is seen after intrastriatal administration of DA on one side (UNGERSTEDT, BUTCHER, BUTCHER, ANDÉN & FUXE 1969). After the injection of 3 4-dihydroxyphenylalanine or nialamide plus reserpine to rats with a unilateral striatotomy the head and tail are also turned from the side with the highest DA concentration (ANDÉN, DAHLSTRÖM, FUXE & LARSSON 1966). The asymmetry was probably not due to a lesion effect by the metatyramine since the asymmetry disappeared after a second dose of reserpine, concomitantly with the difference in DA content between the two sides.

The asymmetries may also have been produced by the higher amount of metatyramine on the right than on the left side. Metatyramine may serve as a false transmitter and thus, may cause a greater facilitation of the DA neuro-

transmission on the right side. Such an effect is not very probable, however since similar asymmetries were observed for both small and large side differences in the metatyramine content between the two sides. Furthermore, the asymmetries observed during the first hours of the infusion, before and shortly after reserpine treatment, were less pronounced than 24 hours after reserpine, even though the concentration of metatyramine at this time was higher On the other hand, there seemed to be a correlation between the difference in DA between the two sides and the extent of the asymmetries. The remaining DA is probably released by the nerve impulses in a normal way Local treatment with metatyramine plus systemic injection of reserpine may thus, disclose the normal function of the catecholamine neurons in the different regions of the brain.

Acknowledgements

This work was supported by the Swedish Medical Research Council (B70-14X 502-06B B70-14X 715-05B) "Svenska livförsäkringsbolags nämnd för medicinsk forskning "Magnus Bergwalls Stiftelse For generous gifts of drugs we thank Dr A. Pletscher F Hoffmann La Roche, Basel (metatyramine = Ro 4-4657) Diwag Chemische Fabriken AG Berlin (β-hydroxy metatyramine = novadral®) and the Swedish Ciba Ltd., Stockholm (reserpine). We thank Mrs. Karin Andreasson, Mrs. Mirta Baldina, Miss Inger Karlsson, Miss Berit Nilsson and Miss Agneta Westerholm for valuable technical assistance.

REFERENCES

Andén, N.-E.: On the mechanism of noradrenaline depletion by α-methyl metatyrosine and metaraminol. *Acta pharmacol. et toxicol.* 1964, 21, 260–271

Andén, N.-E., A. Dahlström, K. Fuxe & K. Larsson. Functional rol of th nigro-neostriatal dopamine neurons. *Acta pharmacol et toxicol* 1966, 24 263–274.

Bertler A., A. Carlsson & E. Rosengren. A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. *Acta physiol scand.* 1958, 44 273–292.

Carlsson, A. & M. Lindqvist: *I vivo* decarboxylation of α-methyl DOPA and α-methyl metatyrosine. *Acta physiol scand.* 1962, 54 87–94

Carlsson, A. & M. Lindqvist: Metatyramine as a tool for selective protection of catecholamine stores against reserpine. *Europ J Pharmacol* 1967 2, 187–192.

Carlsson, A. & B. Waldeck. A fluorimetric method for the determination of dopamine (3-hydroxytyramine). *Acta physiol scand.* 1958, 44 293–298.

Corrodi, H. & G Jonsson. Fluorescenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 6. Identifizierung der fluoreszierenden Produkte aus m Hydroxyphenyläthylaminen und Formaldehyd. *Hel chim. Acta* 1966, 49 798–806.

Corrodi, H. & G. Jonsson. The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines. *J Histochem. Cytochem.* 1967 15 65–78.

Dahlström, A. & K. Fuxe: Evidence for the existence of monoamine containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta physiol. scand.* 1964. 62 Suppl. 232, 1–55

Falck, B., N. A. Hillarp, G. Thieme & A. Torp: Fluorescence of catecholamines and related compound condensed with formaldehyde. *J. Histochem. Cytochem.* 1962, 10, 348–354

Grossman, S. P.: Behavioral effects of direct chemical stimulation of central nervous system structures. *Int. J. Neuropharmacol.* 1964. 3, 45–58

Jonsson, G. & M. Ritzén: Microspectrofluorometric identification of metaraminol in sympathetic adrenergic neurons. *Acta physiol. scand.* 1966, 67. 505–513

König, J. F. R. & R. A. Klippel: *The Rat Brain. A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem.* The Williams and Wilkins Co., Baltimore, 1963.

Mitoma, C., H. S. Posner, D. F. Bogdanski & S. Udenfriend: Biochemical and pharmacological studies on *o*-tyrosine and its meta and para analogues. A suggestion concerning phenylketonuria. *J. Pharmacol.* 1957. 120. 188–194

Snedecor, G. W. & W. G. Cochran: *Statistical Methods.* 6th ed., The Iowa State University Press. Ames, Iowa. 1967

Ungerstedt, U., L. L. Butcher, S. G. Butcher, N.-E. Andén & K. Fuxe: Direct chemical stimulation of dopaminergic mechanisms in the neostriatum of the rat. *Brain Research* 1969. 14. 461–471

Acta pharmacol. et toxicol. 1970 28 49–56.

From the Research Laboratories of AB Draco,
Lund, Sweden

Adrenergic Receptors in the Guinea Pig Trachea and Lung

By

Henry Persson and Birgitta Johanson

(Received March 5 1969)

Abstract: Th effect of adrenaline, noradrenaline, and isoprenaline was studied *in vitro on the spirally-cut trachea and in anaesthetized animals in vivo. In the in vitro* studies, all agents caused a relaxation of the tracheal muscles, and th relative potencies were in descending order isoprenaline (142.2), adrenaline (21.9), and noradrenaline (1.0). After pretreatment for 20 minutes with propranolol (> 0.5 μg/ml) or INPEA (> 5 μg/ml) adrenaline and noradrenaline caused a contraction of the tracheal muscles. The effect of noradrenaline was 0.44 times that of adrenaline. Th constrictor effect of adrenaline and noradrenaline was antagonized or completely inhibited by the α-receptor blocking agents phenoxybenzamine and dibenamine in a concentration of 1 5 μg/ml. Isoprenalin did not produce any contraction of the tracheal muscles after pretreatment with propranolol or INPEA. In the *in vivo* studies, th animals were anaesthetized with pentobarbital sodium (50 mg/kg intraperitoneally) Intravenous injections were made into the right jugular vein. Changes in tidal volume were recorded according to the method of Konzett & Rössler. Adrenaline, in the doses 2–4 μg/kg, produced weak bronchoconstriction that was enhanced after pretreatment of the animals with propranolol (2 mg/kg). The bronchoconstrictor effect of adrenaline and noradrenaline was not affected by atropine but was inhibited by phenoxybenzamine or dibenamine (1 2 mg/kg). Isoprenaline in doses up to 0.1 mg/kg did not produce any bronchoconstriction after pretreatment with propranolol. The results obtained suggest that th smooth muscles in the guinea-pig trachea and lung contain both α- and β-receptors, the β-receptor being dominant.

Key-words: Drug receptors – adrenergic receptor blockers – bronchodilator agents – adrenergic receptor stimulants.

According to Ahlquist (1948) the adrenergic receptors are classified as α- and β-receptors.

The guinea-pig trachea has been stated to contain only β-receptors (Foster 1966) whereas the guinea-pig lung has been stated to contain both α- and

β-receptors (NAGASAKA *et al.* 1964, LYNN JAMES 1967). It has also been reported that both kinds of receptors are present in the dog lung (CASTRO DE LA MATA 1962). Recent observations by TAKAGI *et al.* (1967) and CHAL & O'DONNELL (1968), however, indicate that the guinea-pig trachea may contain excitatory α-receptors.

Since the guinea-pig trachea is extensively used in the study of the effects of sympathomimetic agents, it is important to know the nature of the adrenergic receptors in this organ. Results obtained in this study favour the assumption that the guinea-pig trachea and lung contain both α- and β-receptors, which are functionally antagonistic.

Methods

The effect of adrenaline, noradrenaline, and isoprenaline was studied *in vitro* on the spirally cut guinea pig trachea and *in vivo* in the anaesthetized guinea-pig. In all, 45 animals were used. For the *in vitro* studies, the trachea from male guinea-pigs of 400–500 g body weight was excised and cut spirally to a strip about 5 mm wide. The trachea was suspended in an organ bath with Krebs' solution (composition: NaCl 6.9, KCl 0.35, $CaCl_2$ 2 H_2O 0.37, $MgSO_4$ 7 H_2O 0.286, $NaHCO_3$ 2.09, KH_2PO_4 0.16 and glucose 1 g/l). No pilocarpine was added to the solution. The temperature of the bath was kept constant at 37° and the solution was well aerated with a mixture of O_2 (95 %) and CO_2 (5 %). The load was adjusted to 2 g and relaxations and contractions were recorded by means of a Grass force displacement transducer (FT 03) and a Grass polygraph model 5. The cumulative method of ARIENS & DE GROOT (1954) was used. The concentration of the agonist in the bath was increased until a maximum effect was obtained. The negative logarithm of the molar concentration of the agonists producing 50 per cent relaxation of their maximal response (pD_2) was calculated. An antagonist was added to the bath 20 minutes before the addition of the agonist. Where other time intervals were used, they are included in the results.

For the *in vivo* studies, male guinea-pigs of 500–900 g body weight were used. The animals were initially anaesthetized with mebumal sodium, 50 mg/kg intraperitoneally. Intravenous injections were made into the right jugular vein, and the arterial blood pressure was continuously recorded in the left carotid artery. Changes in tidal volume were recorded according to the method of KONZETT & RÖSSLER (1940) by means of a Grass volume transducer (PT 5) and a Grass polygraph model 5. Arterial blood pressure was recorded by means of a Statham pressure transducer (P 23 AC).

Concentrations and doses of adrenaline, noradrenaline, and isoprenaline refer to the base of these agents. Doses and concentrations of other compounds used refer to the salt.

The following compounds were used: l-adrenaline bitartrate, l-noradrenaline bitartrate, isoprenaline sulphate, acetylcholine chloride, atropine hydrochloride, dibenamine hydrochloride, phenoxybenzamine (Bensylytum NFN) hydrochloride, 1-(4-nitrophenyl)-2-isopropylamino ethanol hydrochloride (INPEA), and propranolol (Propranylytum NFN) hydrochloride.

Table 1

Isolated guinea-pig trachea. Relative potencies of adrenaline, noradrenaline, and isoprenaline.
pD_2 is the negative logarithm of the molar concentration of the compound producing 50 per cent relaxation of its maximal response.

Compound	$pD_2 \pm$ S. E. M. moles/l	Number of exp.	Relative potency
l-Noradrenaline ...	5.816 ± 0.055	11	1.0
l-Adrenaline	7.157 ± 0.282	6	21.9
Isoprenaline ...	7.969 ± 0.167	6	142.2

Results

In vitro studies.

Isoprenaline, adrenaline, and noradrenaline relaxed the muscles of the guinea-pig trachea. Table 1 lists the relative potencies of the three agents. In descending order they were isoprenaline > adrenaline > noradrenaline. Pretreatment for 20 minutes with INPEA or propranolol antagonized the effect

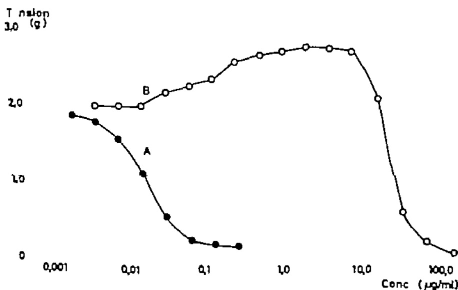


Fig. 1 Isolated guinea-pig trachea. Effect of adrenaline before (A) and after (B) pretreatment with propranolol 1 µg/ml.

4*

of all three agonists and shifted the dose response curve to the right. In the presence of propranolol > 0.5 μg/ml and INPEA > 3 μg/ml, a contraction was obtained for adrenaline and noradrenaline in concentrations below those required for relaxation. Fig. 1 shows the effect of adrenaline before and after pretreatment with propranolol 1.0 μg/ml. As can be seen from the figure, adrenaline within a wide concentration range caused a contraction in the tracheal muscles. The constrictor effect of adrenaline and noradrenaline was antagonized or completely inhibited by the α-receptor blocking agents phenoxybenzamine and dibenamine in a concentration of 1–5 μg/ml. The constrictor effect of adrenaline and noradrenaline was more marked when propranolol was used as the antagonist than when INPEA was used. Adrenaline was more potent in contracting the tracheal muscles than noradrenaline. In 4 experiments where the constrictor effect of these two agents in the presence of propranolol 1 μg/ml was directly compared, the effect of noradrenaline was 0 44 ± 0 07 that of adrenaline (P = 0 01 according to Student's t-test)

In contrast to adrenaline and noradrenaline, isoprenaline did not produce any contractions in the tracheal muscles after pretreatment with propranolol or INPEA.

In vivo studies

Isoprenaline, adrenaline, and noradrenaline are known to cause bronchodilatation in the anaesthetized guinea-pig (for references see FOSTER 1966 LYNN JAMES 1967) and to antagonize bronchoconstriction caused by histamine or acetylcholine. In higher doses, however both adrenaline and noradrenaline caused a bronchoconstriction, adrenaline being more potent in this respect than noradrenaline. Isoprenaline did not produce any bronchoconstriction in doses up to and including 0 1 mg/kg. Higher doses were not studied. Pretreatment with propranolol or INPEA enhanced the bronchoconstrictor effect of both adrenaline and noradrenaline (fig. 2). Atropine in a dose of 5 mg/kg did not antagonize the bronchoconstrictor effect of adrenaline and noradrenaline. The cardiovascular and bronchoconstrictor effects of acetylcholine were abolished by this dose. However the α-receptor blocking agents phenoxybenzamine and dibenamine were effective antagonists (fig. 3).

Discussion

The dual effect of adrenaline and noradrenaline on the muscles of the lung may be well explained by the effect of these agents on two different kinds of adrenergic receptors which are antagonistic in function. A bronchodilatation

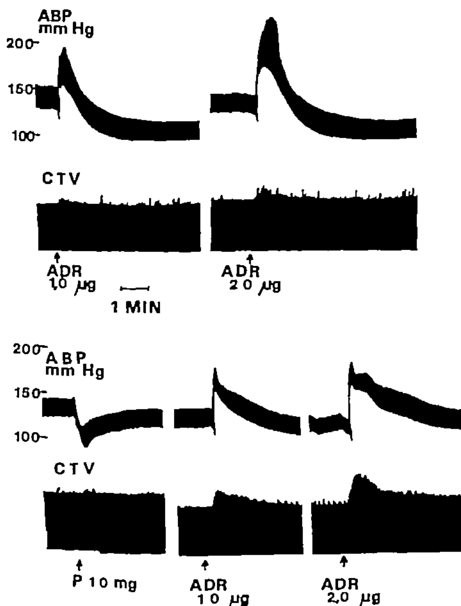


Fig. 2. Guinea-pig (♂) 0.5 kg. Effect of adrenaline (ADR) on tidal volume (CTV = changes in tidal volume) and arterial blood pressure (ABP) before and after treatment with propranolol (P), 1 mg.

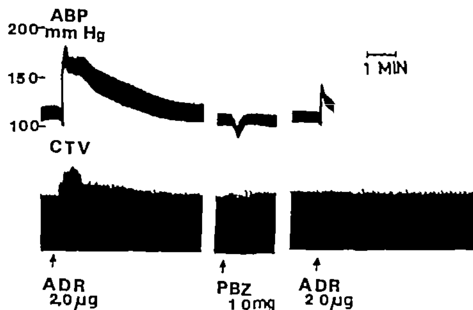


Fig. 3 Guinea pig (♂) 0.5 kg (the same animal as in fig. 2). Th bronchoconstrictor effect of adrenaline (ADR) after pretreatment with propranolol, 1 mg, is completely blocked with phenoxybenzamine (PBZ), 1 mg. Phenoxybenzamine was given intravenously 10 minutes before the injection of adrenaline.

due to the stimulation of the β-receptors and a bronchoconstriction due to the stimulation of the α-receptors.

The relative potencies of adrenaline, noradrenaline, and isoprenaline needed to relax the isolated guinea-pig trachea agree well with those reported by FOSTER (1966) and in descending order are isoprenaline > adrenaline > nor adrenaline. According to AHLQUIST (1948 & 1966) this order indicates a β-receptor stimulation. After blockade of the β-receptors with INPEA or propranolol, adrenaline and noradrenaline caused a contraction of the tracheal muscles. Here the relative potencies of the different compounds in descending order were adrenaline > noradrenaline > isoprenaline, indicating an α-receptor stimulation. Pretreatment with both α- and β-receptor blocking agents antagonized the constrictor effect of adrenaline and noradrenaline. Thus the dual effect of adrenaline and noradrenaline on the isolated guinea-pig trachea can be explained by the effect of these agents on different adre nergic receptors. A relaxation due to β-receptor stimulation and a contraction due to α-receptor stimulation since the results obtained in the *in vivo* studies agree with those obtained *in vitro* the *in vivo* effect cannot be ascribed to circulatory effects of the compounds.

In the guinea-pig ileum, α- as well as β-receptor stimulation causes a

relaxation. According to KOSTERLITZ & WATT (1965) the adrenergic α-receptors in this organ are possibly situated in the cholinergic neurons innervating the longitudinal muscles and have an inhibitory effect on these neurons. In the guinea-pig trachea and lung, the adrenergic α- and β-receptors are antagonistic in function. Stimulation of the α-receptor caused a constriction, whereas β-receptor stimulation caused a dilatation. If the adrenergic α-receptors are situated in the neurons innervating the trachea and bronchial muscles, they must have an excitatory effect on these neurons. Since atropine did not influence the bronchoconstrictor effect induced by adrenaline or noradrenaline, it seems unlikely that the α-receptor would be situated in the neurons in the guinea-pig lung, but rather in the smooth muscles.

The results obtained in this study suggest that the smooth muscles in the guinea-pig trachea and lung contain both α- and β-receptors, the β-receptors being dominant in both organs. Preliminary studies on the human trachea indicate that this too contains both α- and β-receptors (BERTLER, JOHNSON & PERSSON, unpublished).

While this manuscript was being prepared, an article by EVERITT & CAIRNCROSS (1969) appeared. Their results are essentially the same as ours. They worked with *in vitro* technique only. Our study has demonstrated the bronchoconstrictor effect of adrenaline and noradrenaline both *in vitro* and *in vivo*.

REFERENCES

Ahlquist, R. P. A study of the adrenotropic receptors. *Amer. J. Physiol.* 1948, 153, 586–599.

Ahlquist, R. P. The adrenergic receptor. *J. pharm. Sci.* 1966, 55, 359–367.

Ariens, E. J. & W. M. de Groot: Affinity and intrinsic-activity in the theory of competitive inhibition. *Arch. int. Pharmacodyn.* 1954, 99, 193–205.

Castro de la Mata, R., M. Penna & D. M. Aviado. Reversal of sympathomimetic bronchodilation by dichloroisoproterenol. *J. Pharmacol. exp. Ther.* 1962, 135, 197–203.

Chahl, L. A. & S. R. O'Donnell. The actions of orciprenaline and protokylol on guinea-pig trachea. *Brit. J. Pharmacol.* 1968, 33, 552–559.

Daly, I., Catherine De Burgh, O. Hebb & B. Petrovskaia. Adrenaline bronchoconstriction in isolated blood perfused lungs. *Quart. J. exp. Physiol.* 1942, 31, 129–159.

Everitt, B. J. & K. D. Cairncross: Adrenergic receptors in the guinea-pig trachea. *J. Pharm. Pharmacol.* 1969, 21, 97–102.

Foster, R. W. The nature of the adrenergic receptors of the trachea of the guinea pig. *J. Pharm. Pharmacol.* 1966, 18, 1–12.

Konzett, H. & R. Rössler. Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur. *Naunyn-Schmiedeberg Arch. exp. Path. Pharmak.* 1940, 195, 71–74.

Kosterlitz, H. W. & A. J. Watt. Adrenergic receptors in the guinea-pig ileum. *J. Physiol. (Lond.)* 1965, 177, 11P–12P.

Lynn James, G. W. The role of the adrenal glands and of α- and β-adrenergic receptors in bronchodilatation of guinea-pig lungs in vivo. *J. Pharm. Pharmacol.* 1967. 19, 797–802.

Nagamaka, M., J. Bouckaert, A. F. De Schaepdryver & C. Heymans: Adrenergic constriction in isolated guinea-pig lung revealed by Nethalide. *Arch. int. Pharmacodyn.* 1964. 149. 237–242.

Takagi, K., E. Osada, I. Takayanagi & F. Taga. Adrenergic receptors on some organs. *Arch. int. Pharmacodyn.* 1967. 168. 212–219.

Acta pharmacol. et toxicol. 1970, 28, 57–65

From the Research Division of Pharmacia AS, Vanløse,
Copenhagen, Denmark

Evidence for Adrenergic Mediation of Ouabain induced Arrhythmias in the Guinea Pig

By

Kjeld Hermansen

(Received August 6, 1969)

Abstract. Oubain in a dose of 7.5 μg/min. was infused intravenously into urethane anaesthetized guinea pigs weighing from 250–500 g. The incidence of cardiac fibrillation and the cardiotoxic dose (fibrillation, cardiac arrest) were determined in groups of (1) untreated controls, (2) adrenalectomized, (3) reserpinized, 5 mg/kg subcutaneously (4) reserpinized + adrenalectomized, (5) reserpinized + noradrenaline pretreated animals (100 μg/kg intravenously 15 min. before ouabain). All animals were kept at a constant rectal temperature of 34 The pressor response to tyramine (0.1 and 1 mg/kg intravenously) was tested during the same experimental conditions. All animals died in fibrillation except those in (4) in which only 50 % showed fibrillation. The sensitivity to ouabain (minimal fibrillatory dose, LD100) was only slightly decreased in (2) while the LD100 was increased from 239 ± 10 μg/kg t 375 ± 15 μg/kg in (3) and 466 ± 17 μg/kg in (4). Infusion of noradrenaline into reserpinized animals caused partial recovery of the normal ouabain sensitivity as th LD100 decreased from 375 ± 15 μg/kg to 302 ± 11 μg/kg ($P < 0.001$). The pressor response to tyramine showed features almost similar to the ouabain sensitivity except that the sensitivity to tyramine could not be regained by a noradrenaline infusion in reserpinized guinea pigs. The results indicate that the ouabain cardiotoxicity in th guinea pig is at least to some extent mediated through a release of catecholamines and may explain the species dependent difference in potency of some β-adrenergic blocking agents on ouabain arrhythmias.

Key-words: Arrhythmia – catecholamines – ouabain – guinea pigs.

Numerous reports on the influence of adrenergic mechanisms on the cardiac actions of ouabain have been published in recent years. Sufficient data now seem available suggesting that glycosides in some species release cardiac catecholamines, which in turn act upon the myocardium to produce a positive inotropic effect (Tanz 1967).

The mechanism by which the arrhythmogenic effect of cardiac glycosides is

brought about is still obscure and the evidence for an adrenergic mediation of their cardiotoxic effects is quite conflicting. Thus SWAMY *et al.* (1965) found that a continuous infusion of ouabain in the cat did not alter the atrial nor adrenaline content significantly nor did pretreatment with reserpine have any effect on the arrhythmic or lethal dose of ouabain. On the contrary LOUBATIÈRES *et al* (1965) found that in the guinea pig a single dose of ouabain caused a marked depletion of myocardial noradrenaline.

Recently it has been demonstrated (HERMANSEN 1969) that the β-adrenergic blocking agent, 2-isopropylamino-1-(p-nitrophenyl) ethanol HCl (INPEA) though largely devoid of any local anaesthetic effect, is capable of reversing ouabain induced fibrillation in the guinea pig, while in the cat and dog INPEA was found to be ineffective against ouabain induced rhythm disturbances (LEVITT *et al.* 1968 SOMANI *et al* 1965). This observation together with the above mentioned difference in action of ouabain on endogenous noradrenaline in the cat and the guinea pig led to the present study on the mechanism of ouabain induced cardiac fibrillation and lethal effects in the guinea pig. In order to determine the effect of intact adrenergic mechanisms on the cardiotoxic action of ouabain this was evaluated in different experimental conditions such as after adrenalectomy reserpine pretreatment, etc. Furthermore the pressor response to tyramine was measured after the same experimental procedures to see whether the sensitivity to ouabain and tyramine behaved in a similar manner

Material and Methods

Test drugs.

Noradrenalini bitartras, NFN ouabain (g-strophantinum, NFN); reserpine (Aldrich) tyramine monohydrochloride (NBC) and urethanum, NFN The doses mentioned in the text refer to th form of the substances given in this paragraph except for noradrenaline bitartrat which is expressed as the base.

Determination of the ouabain cardiotoxicity

Male guinea pigs weighing from 250–500 g were anaesthetized with urethane (1.5 g/kg intraperitoneally) and given artificial respiration. Ouabain was infused continuously into the jugular vein by means of an infusion pump. The concentration of ouabain was 50 µg/ml and the rate of infusion so that 7.5 µg was given during 60 sec. and not varied according to th weight of the animals. Thus the doses/kg/min. varied from 15 to 30 µg of ouabain. The body weight of the groups (n = 10) varied from 323 to 430 g so that the doses of ouabain from group to group only varied from 17 to 23 µg/kg/min a difference which is unlikely to interfere with the fibrillatory or the lethal dose level of ouabain. The ECG was recorded with bipolar leads. During th infusion of ouabain the typical changes in heart function. bradycardia, ventricular rhythm etc., were observed but only the minimal dose necessary to cause fibrillation and the fatal dose were recorded.

The cardiotoxicity of ouabain was determined in different groups of guinea pigs in which the following experimental procedures were performed 1) no pretreatment before the ouabain infusion (the control group), 2) adrenalectomy between 15 and 30 min. before th ouabain infusion was started, 3) 5 mg/kg reserpine given subcutaneously approximately 16 hrs before, 4) reserpine pretreatment (5 mg/kg subcutaneously) + adrenalectomy and 5) reserpine pretreatment (5 mg/kg subcutaneously) + 100 μg/kg noradrenaline infused at a rate of 1.5 μg/kg/min. In all experiments in which nor adrenaline infusion was performed a 15 min. interval was allowed to elapse between this and the start of the ouabain infusion in order to allow the blood pressure and heart rate to return to th preinfusion level.

T avoid the possibility that differences in body temperature should influence the results, this was kept constant at 34 by external heating. This involved warming the reserpinized animals and cooling the non-reserpinized animals before th ouabain infusion was started. All groups consisted of 10 animals.

Determination f the pressor response to tyramine.

The pressor response to the indirectly acting amine tyramine was taken as a measure of the tyramine releasable pool of noradrenaline. Groups of five male guinea pigs weighing from 310–460 g were anaesthetized with urethane (1.5 g/kg intraperitoneally). After exposure to the same experimental procedures as described above th pressor response to 0.1 and 1 mg/kg tyramine given intravenously was measured by means of Hg-manometer connected to a carotid artery During the experiments the body temperature was kept constant at 34 by means of external heating.

Results

Table 1 shows the effect of the different experimental procedures on the incidence of fibrillation and the minimal dose of ouabain which causes fibrillation and cardiac arrest. As seen from the table all animals in the control group died in fibrillation and the fibrillatory and lethal doses were determined to be 187 ± 9 and 239 ± 10 μg/kg ($\bar{x}$ ± S. E. M.) respectively Adrenalec tomy did not decrease the incidence of fibrillation while the fibrillatory dose level slightly increased to 214 ± 6 μg/kg ($P < 0.05$). The lethal dose of ouabain was not significantly different from that of the control group. Pretreatment with 5 mg/kg reserpine subcutaneously 16 hrs before the ouabain infusion had no influence on the frequency of fibrillations although it caused a considerable decrease in sensitivity to ouabain, resulting in an enhancement of the fibrillatory and lethal dose levels to 309 ± 12 and 375 ± 15 μg/kg ($P < 0.001$) respectively The combined pretreatment with reserpine and adrenalectomy increased the two dose levels still further and this was the only procedure which caused a decrease in the incidence of fibrillation. Only 50 / of the animals in this group died in fibrillation. The remaining animals died in asystole.

Fig. 1 shows two series of ECG recordings, one from a control experiment (section a) and the other from a guinea pig which was pretreated with 5

Table 1

Effect of different experimental procedures on ouabain induced cardiotoxicity in guinea pigs.

Pretreatment	No. of animals	% of animals showing fibrillation	Ouabain $\mu g/kg$ i.v. causing* fibrillation	Ouabain $\mu g/kg$ i.v. causing* cardiac arrest
A No	10	100	187 ± 9	239 ± 10
B Adrenalectomy	10	100	214 ± 6 $P_{BA} < 0.05$	257 ± 8 $P_{BA} > 0.1$
C 5 mg/kg reserpine s.c.	10	100	309 ± 12 $P_{CA} < 0.001$	375 ± 15 $P_{CA} < 0.001$
D 5 mg/kg reserpine s.c. + adrenalectomy	10	50	419 ± 21 (5) $P_{DA}, P_{DB}, P_{DC} < 0.001$	466 ± 17 $P_{DA}, P_{DB}, P_{DC} < 0.001$
E 5 mg/kg reserpine + 100 $\mu g/kg$ noradrenaline i.v.	10	100	249 ± 11 $P_{EC} < 0.005$	302 ± 11 $P_{EC} < 0.001$

* mean ± S.E.M.

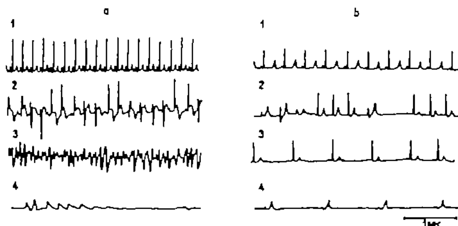


Fig. 1 Effect of a continuous infusion of ouabain on the ECG in an untreated guinea pig (a) and a guinea pig pretreated with reserpine (5 mg/kg subcutaneously) and adrenalectomized before ouabain administration (b). At (1) th control ECG is seen. (2), (3), and (4) show the effect of increasing doses of ouabain. At (4) death occurred. Note the fibrillation in (3a) and the absence of fibrillation in section (b)

mg/kg reserpine subcutaneously 16 hrs and adrenalectomized 15 min. before the infusion of ouabain (section b) The tracings clearly demonstrate that the control animal showed definite fibrillation while the reserpine pretreated and adrenalectomized animal after a period with irregular heart rhythm, extra systole, etc. developed severe bradycardia and died in asystole.

In order to determine whether the decreased sensitivity to ouabain caused by pretreatment with reserpine was due to the noradrenaline depleting effect of the latter compound, a series of reserpinized guinea pigs were given a dose of 100 μg/kg noradrenaline intravenously as a continuous infusion, at a rate of 1.5 μg/kg/min. (experimental group E, table 1) The infusion of nor adrenaline caused a significant increase in sensitivity to ouabain towards the normal. Thus the fibrillatory and the lethal doses decreased from 309 and 375 μg/kg (group C) to 249 and 302 μg/kg (group E) respectively A complete recovery of the normal sensitivity was not obtained by the infusion of noradrenaline.

As mentioned previously the pressor response to tyramine was investigated during the same experimental conditions as the cardiotoxicity of ouabain Table 2 shows the effect of the different pretreatments on the blood pressure level, heart rate and the pressor response to 0 1 and 1 mg/kg tyramine given intravenously As seen from the table the blood pressure and heart rate level was unaffected 15 min. after adrenalectomy had been performed while the tyramine response was considerably reduced. Pretreatment with reserpine (group C) caused an approximately 50 / reduction of the blood pressure level and heart rate as compared to the control group, and almost abolished

Table 2

Effect of different experimental procedures on the pressor response to tyramine in guinea pigs.

Pretreatment	No. of animals	Blood pressure* mm *Hg*	Heart rate beats/min.	Blood pressure increase after injection of	
				0.1 mg/kg tyramine i. v	1 mg/kg tyramine i. v
A No	5	51 ± 2	312 ± 16	10 ± 2	19 ± 1
B Adrenalectomy	5	58 ± 3 $P_{BA} > 0.05$	306 ± 7 $P_{BA} > 0.3$	4 ± 1 $P_{BA} < 0.05$	11 ± 1 $P_{BA} < 0.001$
C 5 mg/kg reserpine s. c.	5	24 ± 2 $P_{CA} < 0.001$	169 ± 18 $P_{CA} < 0.001$	1 ± 0.5 $P_{CA} < 0.001$	4 ± 1 $P_{CA} < 0.001$
D 5 mg/kg reserpine s. c. + adrenalectomy	5	17 ± 2 $P_{DC} < 0.05$ $P_{DA}, P_{DB} < 0.001$	130 ± 9 $P_{DC} > 0.05$ $P_{DA}, P_{DB} < 0.001$	0	0
E 5 mg/kg reserpine s. c. + 100 µg/kg noradrenaline i. v	5	21 ± 2 $P_{EC} > 0.05$	183 ± 20 $P_{EC} > 0.05$	1 ± 0.5 $P_{EC} \sim 0.5$	2 ± 1 $P_{EC} > 0.3$

* mean ± S. E. M.

the pressor response to tyramine. When finally the pretreatment with reser pine was combined with adrenalectomy this reduced the blood pressure and heart rate still further and completely abolished the tyramine response.

When an attempt was made to regain the control level of blood pressure and heart rate and the normal sensitivity to tyramine in the reserpinized group by infusion of the same dose of noradrenaline as used in the ouabain experiments (table 2, group E), this did not affect either the blood pressure and heart rate measured 15 min. after the infusion, or the decreased sensitivity to tyramine. This was in contrast to the effect of the noradrenaline infusion on the decreased sensitivity to ouabain in the reserpinized group (table 1 group C) Here the amine infusion caused a definite increase of the sensitivity towards the normal as mentioned previously

In order to investigate the possibility that the return of sensitivity to ouabain in reserpinized animals as a response to a noradrenaline infusion could be due not to a specific refilling of noradrenaline stores responsible for the cardiotoxic effect of ouabain but to a simple additive toxicity of the two compounds, the following experiment was carried out. To a group of ten untreated guinea pigs 500 μg/kg noradrenaline was infused intravenously in the course of 33 min. Thus the dose per min. was 15 μg/kg or ten times larger than that in the previous experiments. When the dose levels of ouabain necessary to cause fibrillations and death were then determined they were found to be 225 ± 14 and 271 ± 10 μg/kg ouabain respectively The cor responding values in the control group were 187 ± 9 and 239 ± 10 μg/kg ouabain. Thus a noradrenaline dose which was five times larger and an infusion rate ten times faster than that used in the reserpine experiments did not increase the cardiotoxicity of ouabain in normal guinea pigs. This finding is in favour of the view that the partial restoration of the normal sensitivity to ouabain in reserpinized animals by infusion of noradrenaline is due to a specific effect of this amine.

Discussion

The results clearly demonstrate that pretreatment with a large dose of reserpine which causes an almost complete depletion of noradrenaline in the myocardium and peripheral adrenergic nerves considerably decreases the cardiotoxicity of ouabain in the guinea pig. While adrenalectomy alone was ineffective in changing the sensitivity to ouabain the combination of reserpine pretreatment and adrenalectomy evoked the most marked depression of the sensitivity This procedure was also the only effective way of decreasing the incidence of fibrillation during the ouabain infusion. The pressor response to tyramine which was taken as an indirect measure of the

pool of noradrenaline showed some characteristics in common with the ouabain cardiotoxicity. A distinct difference between the response to ouabain and tyramine was however revealed by infusion of noradrenaline. This procedure caused a partial recovery of the normal cardiotoxicity of ouabain in reserpinized animals while the pressor response to tyramine was affected. The latter finding seems incompatible with previous observations (MUSCHOLL 1961) who obtained a restoration of the pressor response to tyramine tested immediately after the cessation of a noradrenaline infusion in the reserpinized rat. In our experiments however the pressor response was tested 15 min. after the noradrenaline infusion at which time tyramine fails to exert a nor adrenaline releasing action (IVERSEN *et al.* 1965). Only after pretreatment with a MAO-inhibitor were we able to restore the tyramine response by a noradrenaline infusion in the reserpinized guinea pig.

It has been shown by many investigators (MUSCHOLL 1960, IVERSEN *et al.* 1965) that noradrenaline infusion into reserpinized animals only leads to a minimal accumulation of the amine in the tissue. The rate of uptake of ^{3}H-noradrenaline shows no difference in perfused hearts from normal and pretreated rats. However the disappearance rate is considerably faster in reser pinized animals probably because reserpine prevents noradrenaline from entering through the membrane of the nerve storage granules. Outside the granules the noradrenaline is rapidly destroyed enzymatically. However the results of IVERSEN *et al.* also showed that after infusion of a large dose of noradrenaline there was still a considerable amount left 15 min. after the end of the infusion that is at the time when the ouabain infusion was started in our experiments. Our data indicate that the noradrenaline after an infusion is situated at a site accessible to ouabain but not to tyramine.

The protective action of reserpine on ouabain induced cardiotoxicity may be due to other factors than the noradrenaline depleting effect. Thus CIOFALO *et al.* (1967) pointed out that the hypotensive, bradycardia producing and hypothermic action of reserpine may all contribute to the decrease in sensitivity to ouabain. In this study a possible effect of hypothermia has been excluded as all experiments were carried out at 34°. That the hypotensive and bradycardic actions of reserpine are of significant importance also seems to be unlikely since the noradrenaline infusion (table 1, group E) caused a considerable increase in sensitivity to ouabain as compared to the reserpinized group C, despite the fact that it did not change either the blood pressure level or the heart rate (table 2, group C and E) at the time when the ouabain infusion was started, i. e. 15 min. after cessation of the noradrenaline infusion. The possibility that the effects observed could be due to a decreased binding of myocardial ouabain in reserpinized guinea pigs, as has been shown for digoxin in the dog and in man (MARCUS *et al.* 1968), is also unlikely because of the action of the noradrenaline infusion. It thus seems justifiable to assume

that it is the depleting effect of reserpine on catecholamine stores which is responsible for the decreased cardiotoxicity of ouabain.

From the results it may be concluded that in the guinea pig, the fibrillatory and lethal effects of ouabain are at least partly brought about by a liberation of catecholamines which then in turn act on the cardiac β-receptors. The finding by LOUBATIÈRES *et al.* (1965) that a single dose of ouabain caused a pronounced fall in myocardial noradrenaline in the guinea pig also supports this view As to the observed species variation in the action of the β-adrenergic blocking compound INPEA on ouabain induced arrhythmias as mentioned in the introduction, this may be explained by assuming that the ouabain cardiotoxicity in the guinea pig is more dependent upon release of catecholamines than in the cat and dog as is suggested by the results of SWAMY *et al.* (1965).

Acknowledgements

The author is most indebted to Mrs. I. Tofte Hyttel for invaluable technical assistance.

REFERENCES

Ciofalo, F B. Levitt & J Roberts Some factors affecting ouabain-induced ventricular arrhythmias in the reserpine treated cat. *Brit. J Pharmacol* 1967 30 143–154

Hermansen, K.. Anti-arrhythmic, local anaesthetic, and adrenergic blocking activity of some β-receptor antagonists. *Brit J Pharmac* 1969 35 476–486.

Iversen, L. L., J Glowinski & J Axelrod. Th uptake and storage of H³-norepinephrine in the reserpine-pretreated rat heart. *J Pharmacol exp Therap* 1965 150 173–183

Levitt, B A. Raines, D. Moros & F G Standaert: Antagonism of ouabain-induced ventricular tachycardia (VT) and death by large doses of N isopropyl-p-nitrophenylethanolamine (INPEA) in the cat. *Fed. Proc* 1968, 27 348.

Loubatières, A., P Bouyard, J Chapal, M. Klein & A. M Rondot: Effets de la g strophanthine sur le taux de catécholamines cardiaques. *Compt Rend. Soc. Biol.* 1965 159 948–950.

Marcus, F L, J Pavlovich, M. Lullin & G Kapadia. The effect of reserpine on the metabolism of tritiated digoxin in th dog and in man. *J Pharmacol exp Therap.* 1968, 159 314–323

Muscholl, E. Die Hemmung der Noradrenalin-Aufnahme des Herzens durch Reserpin und die Wirkung von Tyramin. *Arch. exp Path. Pharmakol* 1960, 240 234–241

Somani, P & Bert K. B Lum. The antiarrhythmic actions of beta adrenergic blocking agents. *J Pharmacol exp. Therap* 1965 147 194–204

Swamy V C., R. L. Hamlin & H. H. Wolf Influence of myocardial catecholamines on the cardiac action of ouabain. *J Pharm Sci.* 1965 54, 1505–1507

Tanz, R. D. Possible relationship between ouabain-induced augmentation and endogenous cardiac catecholamine release. In: *Factors influencing myocardial contractility* Academic Press, New York and London, 1967 563–577

Acta pharmacol. et toxicol 1970 28 66–74

From the Metabolic Division,
Department of Pharmacology University of Gothenburg, Gothenburg, Sweden

Comparison between the Absorption of Nicotinic Acid and Pentaerythritol Tetranicotinate (Perycit®) from Ordinary and Enterocoated Tablets

By

Nils Svedmyr and Lennart Harthon

(Received June 11 1969)

Abstract: Nicotinic acid was shown to be rapidly absorbed from the gastro-intestinal tract but also rapidly eliminated from the blood stream. Administration of nicotinic acid was therefore associated with large fluctuations of nicotinic acid concentration in th plasma. Two alternative ways of maintaining a moderate but more constant increase of the nicotinic acid concentration in the blood were studied. One way was to give nicotinic acid as a sustained release preparation. Nicotinic acid in enterocoated tablets with dissolution times of 2 hrs (in simulated intestinal juice), induced only an irregular and transitory increase of the plasma nicotinic acid concentration after 5 hrs and in some subjects only a reduction in plasma FFA. Enterocoated tablets with a dissolution time of 4 hrs were ineffective. Nicotinic acid is a weak acid and as such is poorly absorbed from the more distal parts of the digestive tract. Therefore the biological prerequisites for this alternative method were not so good. The second alternative method studied was to give nicotinic acid ester which would continuously release nicotinic acid in the body Th nicotinic acid ester used (pentaerythritol tetranicotinate, perycit® Bofors) given in an oral dose of 1 g, was found to give a moderate but consistent and prolonged increase in the free nicotinic acid concentration and a consistent decrease in th FFA-level of plasma with a moderate flush. Nicotinic acid in ordinary tablets and in n equivalent dose induced a considerably more pronounced flush and somewhat larger reduction in plasma FFA which however was of shorter duration. The decrease was accompanied by a secondary and prolonged elevation of the plasma FFA concentration. This secondary increase was not observed in th experiments with pentaerythritol tetranicotinate. The possible importance of these differences from pharmacotherapeutical point of view is discussed.

Key-words. Nicotinic acid – pentaerythritol tetranicotinate – oral absorption.

Nicotinic acid is widely used in the treatment of hyperlipaemic conditions and obstructive vascular diseases. In large doses however it produces side effects such as flushing of the face and neck as well as gastrointestinal symp-

toms (review ALTSCHUL 1964) Orally administered nicotinic acid is rapidly absorbed but is also rapidly eliminated from the blood stream. CARLSON *et al.* (1968) in man observed that 1½–2 hrs after administration of 1 g of nicotinic acid by mouth there was a maximum increase in the plasma level of free nicotinic acid to about 26–28 μg/ml, but after 6 hrs scarcely any nicotinic acid was detectable.

Nicotinic acid depresses the FFA-level in the plasma and CARLSON (1965) has suggested that this is important for the lowering of the triglycerides and cholesterol. After 1 g of nicotinic acid by mouth the plasma FFA level was reduced for only about 3 hrs. It was then followed by a period of marked increase (rebound) over the basal value, being still observable after 6 hrs (CARLSON *et al.* 1968).

In earlier experiments in man (SVEDMYR *et al.* 1969a) we studied the pharmacological effects of an intravenous infusion of nicotinic acid. With a total dose of 150 mg infused during 20 min. the plasma concentration increased to a maximum value of about 4 μg/ml. The pharmacological effects – flush, increased bloodflow in the forearm and hand and decreased FFA-level in the plasma – reached a maximum at the same time. The plasma level of nicotinic acid was no longer elevated and the pharmacological effects had disappeared 40–60 min. after the end of infusion Nicotinic acid was thus rapidly eliminated from the blood stream and inactivated. We found that a concentration of 0 1–0.3 μg/ml nicotinic acid in the plasma significantly increased the blood flow in the hand and forearm and reduced the FFA-level in the plasma. If the plasma concentration of nicotinic acid was further raised to 3–6 μg/ml, the vasodilator action of nicotinic acid in the forearm was significantly increased whereas the actions on the blood flow of the hand and the FFA level in the plasma were not significantly changed. Flushing of the face and upper part of the body was only present as long as the plasma level of nicotinic acid continued to increase but disappeared when the plasma concentration reached a constant, but in other respects active pharmacological level.

It is therefore probable that the most important action of the high doses of nicotinic acid used clinically (3–9 g/day instead of smaller doses) in the treatment of hyperlipaemic conditions is to prolong the duration and prevent the rebound phenomenon without increasing the intensity of its action

It was therefore probable that if nicotinic acid could be administered so as to give a sustained but moderate increase in the plasma level, the pharmacotherapeutic actions might be as good as after larger but fluctuating plasma concentrations, whilst the side effect of flushing could be expected to be reduced. With oral administration of nicotinic acid this goal might be attained in two ways. 1 by giving nicotinic acid in a sustained release preparation which continuously released nicotinic acid in the gastrointestinal tract, 2. by

5*

giving nicotinic acid as an ester compound which after absorption would be gradually hydrolyzed thus releasing nicotinic acid in the body

In the investigation described below we studied the biological prerequisites for these two alternatives. If nicotinic acid is to be pharmacologically active in the sustained release form, it must be comparatively highly absorbable in the more distal part of the small intestine. If a nicotinic acid ester is administered, it must be shown to release free nicotinic acid in the body in a pharmacologically active amount. We studied the first alternative by giving 1 g of nicotinic acid in the form of a) ordinary tablets à 0.5 g and b) enterocoated tablets à 0.2 g with dissolution times of 2 and 4 hrs in simulated intestinal juice (U. S. P.) and observed how these two different tablets influenced the concentration of free nicotinic acid and free fatty acids (FFA) in the plasma. The change in FFA is a sensitive indicator of the pharmacological effect of nicotinic acid (SVEDMYR *et al.* 1969a). The blood glucose concentration was also determined.

The second alternative was studied by giving 1 g of a nicotinic acid ester (pentaerythritol tetranicotinate à 0.5 g; perycit® Bofors) by mouth, observing how this drug influenced the concentrations of free nicotinic acid and FFA in the plasma. In order to ascertain if the observed sustained increase in the plasma level of free nicotinic acid after pentaerythritol tetranicotinate was caused by absorption of the compounds in the more distal parts of the gastrointestinal tracts, the nicotinic acid ester was also given in the form of enterocoated tablets à 0.2 g.

Methods

The experiments were performed on 5 healthy male subjects aged 25–32 who participated in all the series of experiments. The volunteers wh had fasted since the previous evening, were placed in a thermostatically controlled room (+ 25 ± 0.5°) during the experiments. The tablets were given with orange juice. Blood samples were drawn through catheter inserted into cubital vein.

Two basal blood samples were first taken, after a rest period of 60 min., for the determination of the plasma nicotinic acid, FFA and glucose concentrations. 1 g of nicotinic acid or of pentaerythritol tetranicotinate was then given and further blood samples were taken after 15, 30, 60, 120, 180, 240, 300 and 360 min., and in some experiments also after 420 min. The experiments were repeated with other forms of tablets after intervals of 5–7 days.

The concentration of free nicotinic acid in the plasma was determined according to CARLSON (1966). The "blank extinction" given by this method was subtracted (SVEDMYR *et al.* 1969a). The total pyridine compounds of whole blood were determined according to HARTHON *et al.* (1964). The content of FFA in th plasma was determined according to TROUT *et al.* (1960) and the blood glucose according to BERGMEYER & BERNT (1962).

Results

Nicotinic acid.

Nicotinic acid in ordinary tablets induced a pronounced flush. A large and rapid increase in the plasma nicotinic acid concentration occurred, which during the period 30–60 min. after ingestion reached a mean maximum value

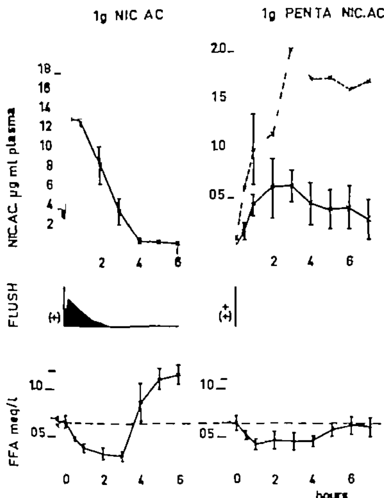


Fig. 1 Effect of 1 g nicotinic acid in ordinary tablets à 0.5 g (nic. ac.) or 1 g penta erythritol tetranicotinate in ordinary tablets à 0.5 g (penta nic. ac.) on. 1 The free nicotinic acid concentration in plasma (x–x) or total pyridine compounds in whole blood (x x x), the mean initial content of total pyridine compounds in whole blood was 4.4 ± 0.20 µg/ml. Observ the differences in the scale of the ordinates. 2. The degree of flushing in the face and neck, + + = pronounced flushing, + = moderate flushing, (+) = weak flushing. 3. The content of FFA in meq/l in plasma. Mean ± S. E. M., number of tests = 5.

of 13.5 ± 4.9 μg/ml over the initial value (fig. 1) The maximum increases varied between 10 and 32 μg/ml in the individual cases. The nicotinic acid level then fell rapidly and only insignificantly exceeded the initial value after 5 hrs.

Nicotinic acid in ordinary tablets induced a considerable reduction in the concentration of FFA (fig. 1), which reached a minimum after 180 min. This reduction was − 0.35 ± 0.05 meq/l ($P < 0.001$) in relation to the basal values. The reduction after 240 min. was followed by a significant increase (0.49 ± 0.10 $P < 0.01$) in the FFA concentration above the initial level, and persisted throughout the experiment. This increase in the plasma FFA content occurred at a time when the nicotinic acid concentration in the plasma was still elevated (fig. 1)

When given in the form of enterocoated tablets with a 2 hrs dissolution time in simulated intestinal juice a moderate and transient increase in free

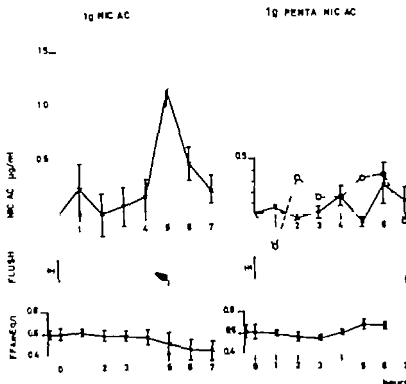


Fig. 2. Effect of 1 g nicotinic acid or 1 g pentaerythritol tetranicotinat in enterocoated tablets à 0.2 g with 2 hrs dissolution time on. 1 The content of free nicotinic acid in plasma (x–x) and total content of pyridine compounds in whole blood (o   o); 2. Flushing of the face or neck. 3. FFA content in plasma, mean ± S. E. M n = 5. See further text to fig. 1

nicotinic acid concentration occurred and two of the patients had a definite increase while the mean increase amounted to about 1 μg/ml after 5 hrs (fig. 2). This was associated with a weak flush in the same two subjects and a marked fall in the FFA-level of plasma in one of the subjects while in the other no consistent effect was observed, the mean decrease in FFA being 0 15 ± 0.09 meq /l. Tablets with 4 hrs dissolution time did not influence the nicotinic acid level in the plasma and had no pharmacological effects.

The blood glucose was not definitely affected by nicotinic acid tablets in any of the experimental series.

Pentaerythritol tetranicotinate

In ordinary tablets 1 g pentaerythritol tetranicotinate induced a moderate but significant increase in the plasma free nicotinic acid concentration in all the subjects after 180 min. It reached a maximum mean value of 0 65 ± 0.20 μg/ml (fig. 1). After 6 hrs the level of nicotinic acid in the plasma was still elevated. The increase in the plasma nicotinic acid concentration was some what greater than that induced by an intravenous infusion of nicotinic acid in a dose of 0.01 mg/kg/min. (SVEDMYR *et al.* 1969a) It was associated with a moderate flush in all subjects. There was an increase of the total pyridine compounds in the blood. This was 3–4 times greater than that of the free nicotinic acid content in the plasma, indicating the presence of pyridine com pounds other than free nicotinic acid (fig. 1). In experiments on the cat, some of these compounds have been identified as pentaerythritol tetranicotinate (BRATTSAND & HARTHON 1969).

In ordinary tablets 1 g pentaerythritol tetranicotinate induced a significant reduction in all subjects (mean maximum reduction 0.24 ± 0 06 meq /l, $P < 0.02$) of the plasma FFA concentration below the basal level, the low values persisting for 4 hrs. After 6 hrs the plasma FFA had regained the basal level. No secondary increase in the plasma FFA was observed.

In enterocoated tablets pentaerythritol tetranicotinate induced no significant increase in the plasma nicotinic acid concentration or in the total content of pyridine compounds in the blood. Neither did it induce any pharmacological effects (fig. 2).

The blood glucose was not affected by pentaerythritol tetranicotinate either in the ordinary or in the enterocoated tablets.

Discussion

In the intestinal tract weak acids and bases are absorbed mainly in an undissociated form (review SCHANKER 1962). For this reason nicotinic acid can be expected to be absorbed mainly in the stomach and upper part of the duodenum, where the pH is sufficiently low to depress the ionisation of the

carboxyl group (pKa = 4.85). The biological prerequisites are relatively weak for the successful administration of nicotinic acid in the form of a sustained release preparation intended to be absorbed in the small intestine. Our results with nicotinic acid in enterocoated tablets give some support to this assumption. In enterocoated tablets nicotinic acid was irregularly absorbed and induced a pharmacological effect in only some of the subjects and there was considerable variation in the individual response.

There is however evidence that β-pyridyl-carbinol which is metabolized in the liver to nicotinic acid can be absorbed from a "sustained release" preparation (RAAFLAUB 1967). The difference between the intestinal absorbance of β-pyridyl-carbinol and nicotinic acid may be attributed to the fact that the former has no carboxyl group dissociable in neutral or an alkaline environment.

The second alternative for inducing a moderate but prolonged increase of the nicotinic acid concentration in plasma was to give it as an ester which would gradually release nicotinic acid in the body. Our experiments with pentaerythritol tetranicotinate in ordinary tablets indicate that this alternative has a better biological prerequisite. Thus 1 g pentaerythritol tetranicotinate in ordinary tablets gave an increase in the plasma nicotinic acid concentration in all tested subjects which was greater but otherwise similar to that attained by a continuous intravenous infusion of nicotinic acid in a dose of 0.01 mg/kg/min. (SVEDMYR *et al.* 1969a) The elevated values in the plasma nicotinic acid concentration persisted for 5–6 hrs after ingestion. Pentaerythritol tetranicotinate was not absorbed from the more distal parts of the gastrointestinal tract. It is therefore probable that the sustained increase in the plasma level of nicotinic acid produced by this compound was caused by absorption of the whole ester which was subsequently hydrolyzed in the body. The increase in the nicotinic acid level was accompanied by a significant reduction in the plasma FFA in all subjects. The decrease was not much smaller than that induced by 1 g nicotinic acid. The increase of nicotinic acid concentration in plasma was however much more moderate than after the administration of nicotinic acid. In another study (SVEDMYR *et al.* 1969b) we also demonstrated that 1 g pentaerythritol tetranicotinate increased the blood flow in cutaneous and muscle tissue and we have also given further support for the assumption that the compound is absorbed as a whole molecule and subsequently hydrolyzed in the tissue. The capacity of human plasma and rat liver homogenates to hydrolyze pentaerythritol tetranicotinate has also been demonstrated *in vitro* (HARTHON unpublished)

One dissimilarity between nicotinic acid and pentaerythritol tetranicotinate in ordinary tablets was that the former induced a secondary increase in plasma FFA, while the latter did not. This stimulating effect of nicotinic acid on the lipid metabolism has been observed previously by CARLSON & ORÖ (1962)

and is probably caused by a reflex liberation of growth hormone which has a lipolytic effect (IRIE *et al* 1967). CARLSON (1965) considered that the reducing effect of nicotinic acid on the plasma FFA concentration could also explain its effect in decreasing the triglyceride and cholesterol concentrations in blood. If this suggestion is correct the rebound of FFA is very likely not a desirable therapeutic effect (CARLSON *et al.* 1968) In experimental cholesterol-induced hypercholesterolaemia and atherosclerosis in the rabbit, BRATTSAND & LUNDHOLM (1969) found that pentaerythritol tetranicotinate in an equivalent dose had a significantly greater prophylactic effect than nicotinic acid. SIGROTH (1968) also found, on treatment of hypercholesterolaemic patients with pentaerythritol tetranicotinate, that the compound was a potent cholesterolreducing agent. The stronger lipid-reducing effect of pentaerythritol tetranicotinate in relation to that of nicotinic acid in the same dose referred to in the two last papers may be ascribed to the fact that pentaerythritol tetranicotinate produces a more prolonged reduction of the FFA-level in the plasma but no secondary stimulation of the lipid mobilization as in the case of nicotinic acid.

Acknowledgements

We are indebted to Mr Theodore Dzedin and Mrs. Helena Andersson for their assistance. The work was supported by the Swedish Medical Research Council (K67–14X–101–03 and B69–14X–2546–01) and from Läkemedelsindustriföreningen, Stockholm, Sweden.

REFERENCES

Altschul, R., Influence of nicotinic acid on hypercholesteremia and hyperlipemia and on the course of atherosclerosis. In. *Niacin in vascular disorders and hyperlipemia.* (R. Altschul Ed.), Thomas Springfield 1964, p. 3–135

Bergmeyer H. & E. Bernt. D-Glucose Bestimmung mit Glucose-Oxydase und Peroxydase. In. *Methoden der Enzymatischen Analyse.* (H U Bergmeyer Ed.) Verlag Chemie Weinheim 1962, p. 123–130.

Brattsand, R. & L. Harthon: Resorbtion, metabolism and excretion of ^{14}C-pentaerythritol tetranicotinate (Perycit® Bofors) after oral administration to cats. *Arzneimittel-forsch.* 1969 In press.

Brattsand, R. & L. Lundholm. Th effect of nicotinic acid nd pentaerythritoltetranicotinate upon experimental therosclerosis in the rabbit. *J Atheroscler Res* 1969 in press.

Carlson, L. A., Inhibition of the mobilization of free fatty acids from adipose tissue *Ann N Y Acad Sci.* 1965 131 118–142.

Carlson, L. A., Determination of free nicotinic acid in blood plasma. *Clin. Chim. A ta.* 1966, 13, 349-351.

Carlson, L. A. & L. Orö The effect of nicotinic acid on the plasma free fatty acids *Acta Med. Scand.* 1962, 172, 641–645

Carlson, L. A., L. Orö & J Östman Effect of single dose of nicotinic acid on plasma lipids in patients with hyperlipoproteinemia *Acta Med Scand.* 1968, 183, 457–465

Harthon, L., J. J. E. Sigroth & A. Sjöborn. Eine Beitrag zur Klärung der Resorption des Hexanicotinsäureesters des meso-Inositols in Organismus. *Arzneimittelforsch.* 1964. 14. 126–128

Irie, M., M. Sakurno, T. Tsuskima, K. Shizume & K. Nakao. Effect of nicotinic acid administration on plasma growth hormone concentrations. *Proceed. exp. Biol. Med.* 1967. 126. 708–711

Raaflaub, J. Zur Pharmakokinetik von Ronicol retard. *Med. Pharmacol. exp.* 1967. 16. 393–398.

Schanker, L. S., Passage of drugs across body membranes. *Pharmacol. Rev.* 1962. 14. 501–530.

Sigroth, K. Pentaerythritoltetranicotinate (Perycit®) in the treatment of hypercholesterolaemia. *Acta Med. Scand.* 1968. 184. 269–273

Svedmyr, N., L. Harthon & L. Lundholm. The relationship between the plasma concentration of free nicotinic acid and some of its pharmacological effects in man. *Clin. Pharmacol. & Therap.* 1969a. 10. 559–570.

Svedmyr, N., L. Harthon & L. Lundholm. A study of some acute pharmacodynamic effects of pentaerythritoltetranicotinate in man. *Pharmacol. Clin.* 1969b. In press.

Trout, D. L., E. H. Estes & S. J. Friedberg. Titration of free fatty acids of plasma. A study of current methods and a new modification. *J. Lipid. Res.* 1960. 1. 199–201

Acta pharmacol. et toxicol. 1970 28 75–80

From the Department of Pharmacology Royal Veterinary College,
Stockholm, Sweden

Effect of Nicotine Treatment on the Metabolism of Nicotine in the Mouse Liver in Vitro

By

Torbjörn Stålhandske and Premysl Slanina

(Received August 6, 1969)

Abstract. ^{14}C-nicotine was incubated with 10000 × g supernatant fraction of liver homogenate from mice pretreated with nicotine for 3 10 and 17 days respectively The rate of metabolism was measured by th determination of the oxidative nicotine metabolite cotinine. After intraperitoneal injections of 5 mg/kg of nicotine three times daily for three days a 50 % significant decrease in metabolism was observed. A 71 % significant decrease in hepatic glycogen was also seen. Giving nicotine 27.8 and 24.5 mg/kg/24 hours in drinking water for 10 and 17 days respectively did not significantly change the metabolism or th hepatic glycogen levels.

Key-words: Nicotine metabolism.

Only a limited number of reports on the effects of nicotine treatment on its own metabolism in the liver have been published. TAKEUCHI *et al* (1954) reported that the ability of livers from untreated and chronically nicotine treated rats to detoxify nicotine did not differ significantly WERLE & MÜLLER (1941) in similar experiments failed to show any increased detoxication rate in nicotine treated animals In fact, in comparison with untreated animals detoxication was even decreased. However WERLE & USCHOLD (1948) in a later study showed that the detoxifying capacity of the livers in nicotine treated rats had increased a little. The authors concluded that the increased detoxication capac ity for nicotine, developed through its chronic administration, was limited.

The purpose of the present work was to investigate the effects of nicotine treatment on nicotine metabolism in mouse liver The mouse has been chosen as the experimental animal since most of the previous studies in this laboratory concerning the biotransformation of nicotine were performed in this species.

The metabolism of nicotine was measured by the determination of formed cotinine, the major metabolite of nicotine in the mouse (HANSSON *et al.* 1964). A 10000 × g supernatant fraction of mouse liver homogenate was used as a source of liver enzymes (STÅLHANDSKE 1969).

Material and Methods

Compounds

Nicotine-methyl-^{14}C was synthesized according to the method by McKENNIS *et al.* (1962) as described by HANSSON & SCHMITERLÖW (1962). The specific activity of different syntheses varied between 28 and 46 μci/mg. Nicotine-bitartrate was kindly donated by Svenska T baks AB.

Treatment of animals.

Male albino mice of the NMRI strain, weighing 18–20 g, were used. One group of animals was injected intraperitoneally with nicotine-bitartrate dissolved in distilled water in an amount corresponding to 5 mg/kg of the pure nicotine base. The injected volume was 0.09–0.1 ml. This amount of nicotine was injected three times a day at intervals of 4 hours for three days. The controls were simultaneously injected with distilled water. The time between nicotine injections was based on data on the elimination of nicotine in the mouse described by SCHMITERLÖW *et al.* (1967). Two other groups also treated with nicotine received as drinking water a tap water solution of nicotine-bitartrate, in a concentration corresponding to 123 mg/1000 ml of the pure nicotine base, for 10 and 17 days respectively. The amount of nicotine consumed is presented as an average of the daily intak during the period of treatment.

The last injection of nicotine was given 18–20 hours prior to sacrifice and removal of the livers. At the same time the nicotine containing water was exchanged by tap water and all the food was removed.

I vitro studies

The preparation of 10000 × g liver supernatants, th content of the incubation mixtures and the methods used for th determination of the reaction rates have been described previously (STÅLHANDSKE 1969). To the incubation mixture nicotine was added in a concentration of 1.6 mM and under the assay conditions used the enzymatic reaction was linear with time and protein content.

Protein estimation.

The protein concentration of the supernatants was determined by th Biuret method (SZARKOWSKA & KLINGENBERG 1963). Crystalline serum albumine was used as a standard.

Glycogen estimation

A sample of liver homogenate prepared from three livers and used for preparing th 10000 × g supernatant fraction was added to 30 % (w/v) KOH solutions as soon as possible after the animals had been killed. Liver glycogen was determined using the phenolsulphuric acid method of MONTGOMERY (1957).

Statistical evaluation of data.

Student's "t" distribution was used a a test of the null hypothesis. The level of significance used was P less than 0.05.

Results

Results presented in table 1 show that intraperitoneal injection of 5 mg/kg nicotine three times a day for three days significantly inhibited the ability of the mouse livers to metabolize nicotine to cotinine *in vitro*. The treatment also caused a significant decrease in the hepatic glycogen level.

Nicotine 27.8 and 24.5 mg/kg/24 hours administered in drinking water for 10 and 17 days increased the formation of cotinine by 19 and 12 per cent respectively. However, the increase was not significant. No significant effect on the hepatic glycogen level was observed (table 1).

None of the treatments affected the ratio of liver weight to body weight. The amount of water consumed by control and experimental animals was approximately the same. No clinical effects of nicotine were observed in mice treated with nicotine in drinking water.

Discussion

Repeated intraperitoneal injections of nicotine (15 mg/kg/24 hours) for three days significantly impaired the metabolism of nicotine in mouse liver *in vitro*. Contrary to this, animals treated with nicotine in drinking water for 10 and 17 days (27.8 and 24.5 mg/kg/24 hours respectively) showed a slightly increased metabolism of nicotine. However, the increase was not significant and the stimulating effects of nicotine on hepatic metabolism under the experimental conditions used, must be considered to be limited.

The dual effects of nicotine treatment on its hepatic metabolism observed in the present investigation is substantially in agreement with the observations of WERLE & USCHOLD (1948) and WERLE & MULLER (1941).

A stimulating and an inhibiting effect of nicotine treatment on hepatic drug metabolism was also observed by YAMAMOTO *et al.* (1965) who found that after daily intraperitoneal injections of 40 mg/kg of nicotine in corn oil for 2 and 3 days, an inhibition of the hepatic metabolism in rats of 2-acetyl-aminofluorene was observed. After a single administration of doses ranging from 5 to 40 mg/kg of nicotine 24 hours before the metabolic assays, however, there was a stimulation of 2-acetyl-amino-fluorene metabolism.

In addition, WENZEL & BROADIE (1966) who gave mice nicotine in drinking water (2.28 mg/kg/24 hours) for two days observed a stimulation of hepatic meprobamate metabolism.

Considering the above mentioned observations it is evident that nicotine may both stimulate and inhibit hepatic metabolism of tested drugs. The kind of effect produced is very likely dependent of the dose and the duration of treatment.

Table 1

Effects of nicotine treatment on the liver metabolism of nicotine to cotinine *in vitro*, hepatic glycogen levels and the ratio of liver weight to body weight. Results are presented as means ± standard error. From each pool of three livers, three incubations and two glycogen determinations were made. Number of liver pools are shown in brackets.

Pretreatments	No. of mice	Liver Wt./Body Wt. × 10²	Hepatic glycogen levels (%)	% change from controls	No. of assays	Cotinine formed µmol/hr/100 mg protein	% change from controls	No of assays
A. Controls (water inject one)	12	5 37 ± 0 05	0.41 ± 0.04		8 (4)	1 92 ± 0 05		12 (4)
Nicotine 5 mg/kg × 3 daily for 3 days injected intraperitoneally	12	5 09 ± 0.13	0 12 ± 0.01	− 71 P < 0.001	8 (4)	0.96 ± 0.05	− 50 P < 0.001	12 (4)
B. Controls	9	5.21 ± 0.15	0.45 ± 0.18		6 (3)	2.68 ± 0.16		9 (3)
Nicotine in drinking water for 10 days. Average dose 27.8 mg/kg/24 hours.	9	5.22 ± 0 32	0 43 ± 0 08	− 4	6 (3)	3 19 ± 0.05	+ 19	9 (3)
C. Controls — — —	9	5.29 ± 0.06	0.36 ± 0 04		6 (3)	2.55 ± 0.18		9 (3)
Nicotine in drinking water for 17 days. Average dose 24.5 mg/kg/24 hours — —	9	5.03 ± 0 09	0.70 ± 0.11	+ 100 P > 0.05	6 (3)	2 87 ± 0.23	+ 12	9 (3)

The repeated intraperitoneal injections of nicotine for three days which significantly impaired the *in vitro* metabolism of nicotine also lowered the hepatic glycogen level

FOUTS (1965) stated that in nearly all cases of decreased rate of hepatic drug metabolism studied, it was also possible to show a decreased level of hepatic glycogen. As a consequence of this he suggested that the process of glycogen storage or maintenance of storage was related to the ability of the liver to metabolize drugs.

It is known that nicotine interferes with hepatic glycogen storage causing glycogenolysis (KOBAYASHI 1938) and hyperglycemia (for review see LARSON *et al.* 1961). The most important component in this mechanism is very likely a direct effect of nicotine on the adrenals where nicotine stimulates the release of catecholamines (for review see LARSON *et al.* 1961 & 1968)

FOUTS (1965) has shown that injections of epinephrine (adrenalinum NFN) or norepinephrine (noradrenalinum NFN) in amounts producing a depletion of hepatic glycogen, could significantly suppress the *in vitro* metabolism of some drugs, i. e. hexobarbital (enhexymalum NFN) One may therefore postulate that the glycogenolytic effect of nicotine mediated through catecholamines is closely associated with the inhibition of nicotine metabolism observed. This assumption is also supported by the fact that when nicotine was administered to the drinking water for 10 or 17 days and no decrease of glycogen was observed there was no inhibition of nicotine metabolism.

WESTFALL (1965) suggested that the adrenals gradually become tolerant to repeated administration of nicotine. They observed that the stimulation of urinary excretion of catecholamines observed after daily injections of 1 mg/kg nicotine in rats increased progressively and reached a peak after three days. This initial increase became gradually less during prolonged administration of the drug and fell off sharply by day 7

In addition KOBAYASHI (1938) reported that the initial increase of epinephrine in blood observed after nicotine injection disappeared after chronic nicotine administration. He also observed that the initial effects of nicotine on glycogenolysis and hyperglycemia were absent in chronically nicotine treated rabbits.

The effect of chronic nicotine administration on the excretion of catechola mines may explain why hepatic glycogen depletion was observed in mice treated for three days but not in mice treated for 10 and 17 days and why inhibition of the metabolism only occurred after treatment for three days.

Acknowledgements

This work was supported by grants to Professor Carl G Schmiterlöw from the Swedish Tobacco Company The American Medical Association Edu-

cation and Research Foundation, and Knut och Alice Wallenbergs Stiftelse (for general equipment for isotope research work)

The skilful technical assistance of Mrs. A. Österblom and Mrs. B Hagtom is gratefully acknowledged.

REFERENCES

Fouts, J R.. Impairment of drug metabolism in drug toxicity In. *Drugs and Enzymes.* (Ed. B B Brodie and J R. Gillette) Pergamon Press, 1965 261 276

Hansson, E., P C. Hoffmann & C. G Schmiterlöw· Metabolism of nicotine in mouse tissue slices. *Acta physiol scand* 1964 61 380–392.

Hansson, E. & C. G Schmiterlöw· Physiological disposition and fate of ^{14}C-labelled nicotine in mice and rats. *J Pharmac. exp Ther* 1962, 137 91–102.

Kobayashi, S., Experimentelle Untersuchungen über die Wirkung des Nikotins auf den Kohlehydrothaushalt. *Jap J med. Sci Pharm.* 1938, 11 11–46.

Larson, P S., H. B Haag & H. Silvette· *Tobacco. Experimental and Clinical Studies.* *The Williams & Wilkins Company Baltimore,* USA, 1961

Larson, P S. & H. Silvette: *Tobacco. Experimental and Clinical Studies* Supplement I. The Williams & Wilkins Company Baltimore, USA, 1968.

McKennis, Jr H., L. B Turnbull, S. L. Schwartz, E. Tomaki & E. R. Bowman. Demethylation in the metabolism of ( ) – nicotine. *J biol. Chem.* 1962, 237 541 546.

Montgomery R.. Determination of glycogen. *Arch. Biochem. Biophys.* 1957 67 378–386.

Schmiterlöw C. G E. Hansson, G Andersson, L. E. Appelgren & P C. Hoffmann. Distribution of nicotine in the central nervous system. *Ann. N Y Acad. Sci.* 1967 142, 2–14

Stålhandske, T The metabolism of nicotine and cotinine by a mouse liver preparation *Acta physiol. scand.* 1969 In press.

Szarkowska, L. & M. Klingenberg: On the role of ubiquinone in mitochondria. *Biochem.* Z. 1963, 338, 674–697

Takeuchi, M., Y Kurogochi & M Yamaoka Experiments on the repeated injection of nicotine into albino rats. *Folia pharmac. jap.* 1954 50 66–69

Wenzel, D G & L. L. Broadie: Stimulatory effect of nicotine on th metabolism of Meprobamate. *Toxic appl Pharmac* 1966, 8, 455–459

Werle, E. & R. Müller· Über den Abbau von Nikotin durch tierisches Gewebe. II. *Biochem. Z* 1941 308, 355–358.

Werle, E & E Uschold Über fermentative Nikotinentgiftung durch tierisches Gewebe. *Biochem.* Z. 1947/48.

Westfall, T C Tobacco alkaloids and release of catecholamines. In. *Tobacco Alkaloids and Related Compounds* (Ed. U S. von Euler) Pergamon Press, 1965 4 179–203.

Yamamoto, I., K. Nagai, H. Kimura & K. Iwatsubo: Nicotine and some carcinogens in pecial reference to th hepatic drug-metabolizing enzymes. *Jap J Pharmac.* 1966, 16, 183–190.

Acta pharmacol. et toxicol. 1970, 28, 81–96

From the Research Laboratories of
H. Lundbeck & Co. A/S,
Copenhagen/Valby, Denmark

Metabolism, Distribution and Excretion of the Thiophthalane Lu 5-003, a Bicyclic Thymoleptic

By

K. Fredricson Overø, A. Jørgensen and V. Hansen

(Received July 24, 1969)

Abstract. The metabolism of orally administered Lu 5–003 has been studied in the rat, dog and man. Urine and faeces were studied by thin layer chromatography of basic and acidic extracts. The metabolic degradation was shown to proceed partly by sulphoxidation and partly by demethylation followed by deamination of the side chain. Seven metabolites were found. The pattern was the same in the three species investigated. A semiquantitative determination showed Lu 5–003 and the demethylated metabolite to be the main excretion products in the faeces, while the more water soluble sulphoxides of these compounds and propionic acid derivatives were dominant in the urine. ^{14}C-labelled Lu 5–003, given intravenously to rats, was used for distribution and excretion studies. Groups of three rats were killed at different times after administration and the amount of radioactivity was determined in the blood and tissues. The distribution was shown to follow the well known pattern of psychotropic drugs, i.e. high concentrations in the lungs, liver, kidneys and heart, while the brain concentration was considerably lower. Lu 5–003 and its demethyl derivative constituted the main part of radioactivity in the organs as shown by thin layer chromatography of basic extracts. Up to 1 hour after administration Lu 5–003 was the main compound in the brain, but this was exceeded later by the demethylated metabolite. The half life of radioactivity in the blood and brain was calculated to be about 2 hours. After a single dose 60 % and 23 % of the radioactivity was excreted in the faeces and urine respectively in 7 days, while 1 % remained in the body. During the first 24 hours 72.6 % was excreted. There was evidence of an enterohepatic circulation. Expired CO_2 showed minute amounts of radioactivity shortly after administration, probably originating from impurities.

Key-words. Thymoleptics – kinetics – chromatography, thin layer – radioisotopes.

The pharmacological properties of a series of bicyclic thymoleptics consisting of phthalanes and thiophthalanes are described by PETERSEN *et al.* (1970). 3,3-Dimethyl-1-(3-methylaminopropyl)-1-phenyl-thiophthalane (Lu 5-003) possessed the strongest thymoleptic properties among the thiophthalanes

as did the corresponding phthalane (Lu 3-010) among the phthalanes. A previous publication dealt with the distribution and metabolism of Lu 3-010 (PLYM FORSHELL *et al.* 1968) and the present paper will describe the metabolism of Lu 5-003 in dogs, humans and rats together with the quantitative aspects of its distribution and excretion in rats.

Materials and methods

A. Studies with unlabelled compound.

1 Dog studies

Studies on the identification of metabolites were performed on urine and faeces collected separately from beagl dogs given a daily dose of 5 or 25 mg/kg orally for 6 months. The samples were collected after administration periods of 24 hours, 1 month and 5 months.

2. Rat studies

Urine and faeces, collected separately from male Wistar rats (100–130 g) given an oral dose of 50 mg/kg daily for 5 days, were used in biotransformation studies.

3 Human studies

Urine and faeces from four female patients under treatment with 60 or 90 mg of Lu 5-003 daily were used. All of the patients had been under treatment for several weeks. Two of the patients supplied 24-hour collections.

4 Preparation of extracts

Extractions were made on urine (10–40 ml) and faeces (1 5 g homogenized in 5 volumes of water) with 3 portions of a doubl volume of dichloroethane at pH 9 followed by extraction at pH 1 Separation of the phases was achieved by centrifugation. The extract was filtered through anhydrous sodium sulphate and evaporated to dryness under vacuum at 40 Th residue was dissolved in 1 or 2 ml of chloroform and used for chromatography

After exhaustive extraction, hydrolysis was undertaken with conc. HCl, β-glucuronidase (Sigma), or *β-glucuronidase/aryl-sulfatase* (C. F Boehringer & Soehne, Mannheim). The samples were again extracted and treated as described above.

All basic compounds (Lu 5-003 Lu 5-054, Lu 5-052, Lu 5-060, Lu 5-106 and Lu 5-105 for formulas see fig. 1 and 2) were completely extractable at basic pH, as were acidic compounds (Lu 5-085 Lu 5-086, Lu 6-107 and Lu 6-099 with sulphoxides for formulas see fig. 1 and 2) at acidic pH. However acidic compounds, especially Lu 5-085, were partly extractable at basic pH. Also, small amounts of basic compounds could be extracted at acidic pH. Therefore, the procedure of basic extraction followed by acidic extraction gave a basic extract ontaining all the basic metabolites and parts of the acidic metabolites and an acidic extract containing acidic metabolites only

Purification of extracts containing many impurities was performed by gel filtration using a Sephadex G 25 column (length 92.5 cm, diameter 1.5 cm). For this procedure the dichloroethane extract mentioned above was evaporated to dryness. The residue was dissolved in 10 ml of distilled water concentrated to bout 1 ml and applied to the

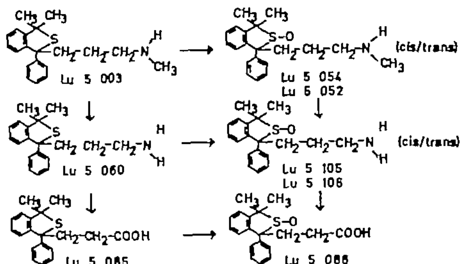


Fig. 1 Biotransformation of Lu 5-003.

column. Elution was performed with 0.1 M sodium phosphate buffer (pH 7.8) and a fixed number of fractions containing a preset volume was collected. The number of fractions and the transmission of the eluate at 254 mμ were recorded automatically. The

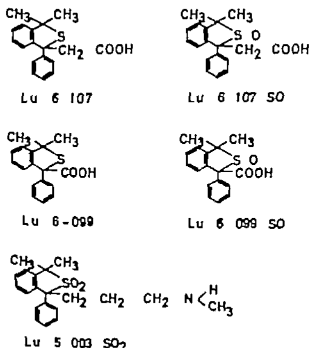


Fig. 2. Some reference compounds.

fractions were pooled in groups of about ten so as to contain only one or two metabolites. Gel filtration of available reference compounds had proved it possible to partly separate all metabolites. The pooled fractions were extracted twice with dichloroethane at a basic or acidic pH. The extracts were treated as mentioned before. In one experiment gel filtration was performed directly on the untreated urine.

5 *Thin layer chromatography (TLC)*

TLC was carried out on glass plates (20 × 20 cm) coated with a 250 μ thick layer of Silica Gel G. according to Stahl (Merck). After coating, the plates were activated at 110° for 30 minutes and stored in a box. Buffered plates were prepared exactly like ordinary plates but with 0.1 M sodium acetate buffer pH 5.4 instead of water. The following solvent systems were used (all unsaturated). Solvent systems 5 and 6 were used only for buffered plates.

1 Cyclohexane: acetone: diethylamine	50:30:5
2. Cyclohexane: acetone: acetic acid	80:20:1
3 Benzene: ethylmethylketone: diethylamine	50:30:5
4 Chloroform. acetone: diethylamine	30:70:1
5 Butanol. water	80:20
6. Cyclohexane: acetone	50:50

Removal of many impurities from impure extracts was carried out using a two-dimensional chromatographic technique. After application of the sample and reference solution to one corner of the plate, development was carried out with a rinsing system which did not elute Lu 5-003 or its metabolites. Chloroform ether (85 15) and benzene: ethylmethylketone (50:30) wer used as rinsing systems. The plate was thoroughly dried and then developed in the second direction with one of the solvent systems mentioned bove.

For the detection of spots the following spray reagents were used (Stahl, 1967)

1 Potassium permanganat – sulphuric acid (all compounds)
2. Ninhydrin (amines).
3 Dragendorff's reagent – Munier & Macheboeuf modification (amines).
4 Bromcresol green (acids).
5 Folin Ciocalteau reagent (phenols).

Spraying with eagent number 1 gave white blue or whit yellow fluorescent spots in UV-light (366 nm). Plates run in basic systems needed short period of heating

The following reference substances were used

Lu 5-003 Lu 5-054 Lu 5-052, Lu 5-060, Lu 5-106, Lu 5 105 Lu 5-085 Lu 5-086, Lu 6-107 and Lu 6-099 (for formulas see fig. 1 and 2). The solvent systems used are able to separate the isomeric forms of the aminesulphoxides, but not of th acid-sulphoxides. Sulphoxides of Lu 6-107 and Lu 6-099 were prepared by direct oxidation on the plate with a small quantity of 10 % H_2O_2-solution.

A semi-quantitative determination of the amounts of Lu 5-003 and its metabolites was made by comparing the siz of the spots with known amounts of standards. This was best accomplished in the rang of 1–6 μg.

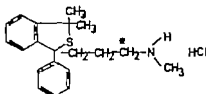


Fig. 3 Lu 5-003-^{14}C. * position of labelling.

B. Studies with labelled compound.

1 Labelled compound.

Lu 5-003-^{14}C was synthesized with the radioactivity in the methylene group alpha to amine nitrogen (Fig. 3). Purity of th substance was controlled by TLC.

Specific activity: 7.7 µci/mg
Radiochemical purity > 94 %

2 Rat studies

a. *Distribution study* Mal Wistar rats (90–110 g) fasted for about 16 hours were given a single intravenous dose of 5 mg Lu 5-003-^{14}C/kg body weight in a tail vein. After different periods of time groups of three animals were killed by exsanguination under ether anaesthesia. Blood was collected in tubes containing EDTA to prevent clotting. Brain, lungs, liver kidneys, heart, spleen, epididymal fat, gastro-intestinal tract, tail and carcass were examined.

b. *Studies on excretion in urine and faeces* In one study 10 male Wistar rats (90–110 g) fasted for bout 16 hours were given 5 mg Lu 5-003-^{14}C/kg body weight by intravenous injection and placed in metabolism cages with food and water ad libitum. Urine and faeces were collected separately for 7 days after administration and examined with th body. In another study 4 male Wistar rats (90-110 g) were given an intravenous dose of 5 mg Lu 5-003-^{14}C/kg body weight daily for eight days. Urine and faeces were collected until 24 hours after th last dose.

c. *Expiration study* Three male Wistar rats (105-115 g) were given an intravenous dose of 5 mg Lu 5-003-^{14}C/kg body weight. The animals were placed in closed box ventilated with atmospheric air which was saturated with moisture. The air from th box was conducted through two bubble flasks. The first one contained 5 % HCl-solution and the second mixture of ethanolaminemethoxyethanol (1.2) in which th expired $^{14}CO_2$ was collected.

d. *Bile collection study* Four male Wistar rats (200-230 g) fasted for about 16 hours were anaesthetized with urethane (0.75 g/kg intraperitoneally and 0.50 g/kg subcutaneously). In two of the animals a polythene tube was inserted into the common bile duct and the bile was collected. Th four animals were given an intravenous dose of 5 mg Lu 5-003-^{14}C/kg body weight, and the bil was again collected from two of the animals. After 130 minutes the animals were killed by exsanguination and th intestinal tract removed. The intestines were opened and th contents washed out in 25 ml of distilled water

3 Preparation of samples.

The scintillators used were toluene-triton X-100 (2:1) containing 6 g/l PPO and 0.3 g/l dimethyl-POPOP for blood samples and the dioxane-methanol-toluene-naphtalene mixture (diotol) described by HERBERG (1960) modified by replacing POPOP with dimethyl-POPOP for all other samples.

a. *Blood.* To 0.1 ml of blood were added 1 ml of conc. NH_3-solution and 0.1 ml of 30 % H_2O_2-solution. After gentle shaking the sample was left at room temperature for one hour and heated for another hour in an oven at 60 . After cooling 10 ml of scintillator were added.

b. *Tissues.* Homogenates containing 1 part by weight in 4 volumes of water were made of brain, lungs, liver, kidneys and spleen. A 0.2 ml aliquot was added to 10 ml of scintillator. Heart, epididymal fat, gastro-intestinal tract and tail were heated in 4 volumes of 0.5 N-NaOH solution in sealed tubes on water bath at 90-95° for 30 minutes with occasional shaking. Aliquots of 0.2 ml were taken for counting in 10 ml of scintillator. The carcass was refluxed for one hour in two volumes of 0.5 M ethanolic KOH-solution. After centrifugation 0.2 ml of the supernatant was counted in 10 ml of scintillator.

c. *Bile, urine and faeces.* 0.05 ml of bile and 0.2 ml of urine were counted directly in 10 ml of scintillator. Each sample of faeces was ground in a mortar to give a homogenous mixture. 500 mg of each sample were heated in an oven at 70-80 for an hour with 1 ml of 60 % $HClO_4$-solution and 2 ml of 30 % H_2O_2-solution. After cooling the solution was neutralized with solid NaOH and mixed with 40 ml of cellosolve. 1 ml of the mixture was counted in 10 ml of scintillator.

4 Extraction and chromatography

Extraction of tissue homogenates, bile, urine and water homogenates of faeces as well as acid hydrolysis was performed as described above under studies with the unlabelled compound except that the extracts were concentrated to about 5 ml. These solutions (0.2 ml) were counted and 0.025–0.2 ml was chromatographed. Basic extracts were chromatographed in systems 2, 3 and 4. Acid extracts were chromatographed in system 1. After development the silica gel was scraped off automatically into separate counting vials for each half centimeter beginning at the starting point and the radioactivity determined in 10 ml of scintillator.

Results

Identification of metabolites

Dog studies. Identification was based on R_f-values in different solvent systems, K_D-values from gel filtration and colour reactions with spray reagents. K_D- and R_f-values for the reference compounds are shown in table 1. All substances gave fluorescent spots with spray reagent no. 1. In addition, the basic metabolites gave colour reactions with spray reagents no. 2 and 3. The acidic metabolites gave faint colour reaction with spray reagent no. 4. Unchanged drug and seven metabolites were identified in urine from dogs. Two

Table 1
K_D and R_f-values of reference substances.

	K_D-value	R_f-values in solvent system					
		1	2	3	4	5	6
Lu 5-003	1 7	0.57	0	0.64	0.30	0.37	0
Lu 5-054	0.91	0.28	0	0.30	0.17	0.13	0
Lu 5-052	1 1	0.23	0	0.24	0.10	0.18	0
Lu 5-060	2.2	0.92	0	0.94	0.86	0.51	0.11
Lu 5-106	1.[illegible]	0 62	0	0.83	0.68	0.31	0.05
Lu 5-105	1 4	0.53	0	0.73	0.54	0.35	0 03
Lu 5-085	2.8	0	0.39	0	0	0 90	0.61
Lu 5-086	1 4-1.6	0	0.13	0	0	0 41	0
Lu 6-107	1.6	0	0.35	0	0	0.79	0.59
Lu 6-107 cr.	0.94-1 1	0	0.16	0	0	0.39	0
Lu 6-099	1 1	0	0.32	0	0	0 62	0.21
Lu 6-099 cr. 1	0 4-0.6 1 1	0	0 05	0	0	0.36 0 42	0
cr. 2	1.3	0	0.32	0	0	0.90	0 69

sulphoxides of Lu 5-003 (the isomers Lu 5-052 and Lu 5-054) were found as well as the primary amine, Lu 5-060 and its sulphoxides, Lu 5-106 and Lu 5-105 Furthermore, the propionic acid derivative, Lu 5-085 and its sulphoxide, Lu 5-086, were identified as metabolites.

In faeces, Lu 5-003 and its sulphoxides were found as well as the deme thylation product, Lu 5-060 Acidic metabolites were not found.

No other metabolites were observed after direct extraction or after hydrolysis. None of the spray reagents revealed other spots than the above mentioned and phenolic metabolites therefore appear to be absent. Hydrolysis did not liberate further amounts of parent compound or metabolites.

The metabolic degradation of Lu 5-003 thus seems to proceed partly by sulphoxidation and partly by demethylation followed by deamination in the side chain. The assumed pathways are shown in fig. 1

The semi-quantitative determination revealed some individual variation in the total concentration of excreted drug and metabolites. A possible explana tion might be differences in the amount of urine and faeces.

However the relative proportions of the metabolites seemed to be rather constant. Tables 2 and 3 show the concentrations of unchanged drug and metabolites in faeces and urine, respectively after 5 months of administration.

In faeces the less water soluble substances Lu 5-003 and the primary amine, Lu 5-060, dominated with each accounting for about 40 / of the total. The remaining 20 / consisted mainly of Lu 5-054 with small amounts of Lu 5-052. The results were similar at both dose levels. The relativel

Table 2

Concentration of Lu 5-003 and metabolites in faeces expressed as $\mu g/g$ Dogs wer given oral doses daily for 5 months.

Dose	5 mg/kg				25 mg/kg			
Dog no. ..	326	336	352	364	314	349	353	363
Lu 5-003 ..	10	24	24	14	40	37	25	25
Lu 5-054	9	13	13	6	35	25	20	5
Lu 5-052 ...	<1	4	3	<1	10	10	<2	<2
Lu 5-060	12	15	16	14	64	70	27	15
Total	31	56	56	34	149	142	72	45

amounts of Lu 5-003 found in faeces may be due to enterohepatic circulation as well as to non absorbed drug.

In urine the more water soluble compounds dominated. Thus, with the high dose only about 9 % and 7 % consisted of Lu 5-003 and Lu 5-060, respec tively while the sulphoxides occupied about 60 % and the acidic metabolites about 35 % of the total. Lu 5-054 followed by Lu 5-085 and Lu 5-086 were the main metabolites with the higher dose. At the lower dose level the acidic metabolites were even more dominant, in two cases amounting to 70 % of total.

One of the two possible isomers of the amine sulphoxides, i. e Lu 5-054 and Lu 5-106 seems to be preferably produced.

Table 3

Concentration of Lu 5-003 and metabolites in urine expressed as $\mu g/ml$. Dogs were given daily oral doses for 5 months.

Dose	5 mg/kg				25 mg/kg			
Dog no	326	336	352	354	314	349	353	363
Lu 5-003	0.6	0.5	0.3	1.0	7.5	26	6 9	14
Lu 5-054	1 6	1.8	1.3	1 5	21	77	28	9.2
Lu 5-052	0 3	< 0.2	0.3	0.7	4.6	16	4.3	3.3
Lu 5-060	< 0 5	ca. 0.4	ca 0 3	< 0 3	8 7	20	7 4	3 0
Lu 5-106	< 1	0 8	0 9	1 0	8 0	25	9 2	6 0
Lu 5-105	1 0	ca 0 3	0.3	0 8	4 1	11	4 0	2.6
Lu 5-085	5.0	1 3	1 3	3 7	13	58	18	8.5
Lu 5-086	7 5	< 0.3	5.3	< 0.3	10	58	18	6 8
Total	17	5	10	9	77	291	96	54

Comparison between collections of urine at different times (24 hours and 1 month) during administration of the higher dose revealed that the proportion of Lu 5-003 diminished with time while the proportion of Lu 5-054 increased. This was not observed with the lower dose. An increased capacity for sulphoxidation was thus indicated No other time dependent changes were observed at either dose level.

Human studies Based on the same criteria as for dog studies, a series of metabolites has been identified in human urine. Lu 5-086 was found to be the main metabolite followed by Lu 5-054 and Lu 5-106 The isomers of the latter compounds, Lu 5-052 and Lu 5 105 were present in smaller amounts. Extremely small concentrations of unchanged drug and of Lu 5-060 were observed.

The sulphoxides of the secondary and primary amines were also identified in faeces. Very small amounts of the parent substance were observed in some cases. No acidic metabolites seemed to be present in the faeces. The presence of another metabolite in urine, presumably the sulphoxide of Lu 6-099 was strongly indicated but its identity could not be established.

Enzyme hydrolysis did not liberate further amounts of drug or metabolites. After acid hydrolysis, however small amounts of Lu 5-003 were liberated, especially from faeces. Also the unidentified metabolite was liberated by acid treatment. This binding is most likely of unspecific nature.

The semi-quantitative determinations showed that individual variations in metabolite pattern were small or absent. In urine the main metabolite, Lu 5-086 was present in concentrations ranging from 8 to 15 μg/ml, while Lu 5-054 and Lu 5-106 amounted to 2–10 / g/ml and 0,5–3 μg/ml, respectively The ratio between the isomers Lu 5-054/Lu 5-052 and Lu 5-106/Lu 5-105 were about 5 1 In faeces about 10–20 / g/g of Lu 5-054 was found with Lu 5-106 amounting to about 5–10 μg/g. The amounts of Lu 5-003 in two patients were less than 5 μg/g and in two others 5–10 μg/g. Thus, there seems to be no reason to doubt good intestinal absorption of the drug. The amounts of drug and metabolites thus recorded in urine and faeces represent about 25 / of the daily dose.

Rat studies. Based on R_f-values, K_D-values and colour reactions with spray reagent no 1 a series of metabolites was identified in the urine and faeces.

Basic urine extracts contained Lu 5-054 as the main metabolite with its isomer Lu 5-052, about $^1/_8$ as plentiful. The demethylated sulphoxide pair Lu 5-106 and Lu 5-105 was also identified. Small amounts of Lu 5-003 and Lu 5-060 seemed to be present. In acidic urine extracts considerable amounts of Lu 5-086, but not Lu 5-085 were identified.

The parent drug Lu 5-003 was identified in faeces together with very small amounts of Lu 5-054 Lu 5-060 was also present, but no acidic metabolites

were observed. The metabolite, supposed to be Lu 6-099-SO which was observed in human samples, could not be detected in rat samples.

A weak brownish coloured spot was observed between Lu 5-003 and Lu 5-054 on the chromatograms. This might be the sulphone of Lu 5-003 (for formula see fig. 2) Further identification was made very difficult by the small amounts present and the impure extracts.

The identification of metabolites was further supported by chromatography of radioactive extracts. Fig. 4 shows examples from chromatograms of basic extracts of urine and faeces. Peaks corresponding to all metabolites identified in studies on rats with unlabelled compound could be seen. Due to the low amount of radioactivity on each chromatogram, not too much attention should be paid to the relative amounts of the different metabolites. Also, more

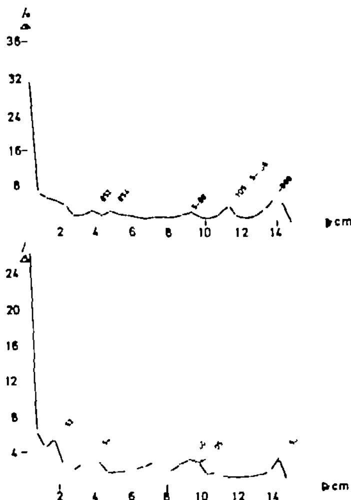


Fig. 4 Radioactivity from chromatograms of basic extracts in system 3 Upper: Urine, 4-8 hours Lower: Faeces, repeated dose

radioactivity remained at the starting point than could be explained by the presence of the acidic metabolites Lu 5-085 and Lu 5-086. This might be explained by disturbances in development caused by impurities in the ex tracts. In acidic extracts from urine and faeces the acidic metabolite Lu 5-085 and its sulphoxide, Lu 5-086, were seen, but as with basic extracts relatively large amounts of radioactivity remained at the starting point. In addition to the identified metabolites the previously mentioned unidentified metabolite was seen in most extracts. From urine 9–19 % of the radioactivity could be extracted at basic pH and 2–12 % at acidic pH. Acid hydrolysis made another 10–25 % extractable at acidic and 6–10 % basic pH. From faeces 40–66 % of the radioactivity could be extracted at basic pH and 6–11 % at acidic pH Following acid hydrolysis 4–12 % could be extracted at acidic as well as basic pH. Thus the total range of extractable radioactivity was 30–52 % in urine and 58–88 % in faeces.

Quantitative aspects of the excretion in rats

Single dose Excretion of radioactivity following a single intravenous dose of Lu 5-003 – ^{14}C is shown in fig. 5 Sixty % of the administered dose was excreted in the faeces and 23 % in the urine in seven days. One % of the dose remained in the animal body The greatest part (72.6 %) of the radioactivity was excreted during the first 24 hours.

Repeated dose Excretion of radioactivity in urine and faeces following a daily dose of Lu 5-003 – ^{14}C for eight days is given in table 4 The amount of a daily dose remaining in the animals 24 hours after the last dose was 14.3 % The low amount of radioactivity remaining in the animals following repeated doses as compared to single dose (see table 5) does not indicate any accumu lation following repeated administration. This assumption is further supported by the almost complete excretion of the daily dose.

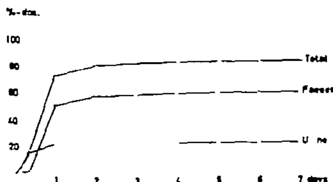


Fig. 5 Cumulative excretion of radioactivity in urine and faeces following single administration of Lu 5-003 – ^{14}C.

Table 4

Excretion of radioactivity following repeated intravenous administration of Lu 5-003 expressed as % of th daily dose. (Pooled samples of 4 rats.)

	Urine	Faeces	Total
0-1 day	27.5	67 4	94.9
1 2 days	26.0	68.8	94.8
3 days	21.3	58.8	80 1
3-4 days	21 4	78.7	100 1
4-5 days ..	19.0	88.3	107.3
5-6 days	20.0	73.3	93.3
6-7 days ..	20.8	88.5	109.3
7-8 days ..	22.3	70.0	92.3

Excretion in bile

Bile collection for 130 minutes from the common bile duct of two rats yielded 1 7 ml of bile containing 29 / of the administered dose. The intestinal contents of these two animals contained radioactivity corresponding to 1.3 and 1.2 / of the dose, while the intestinal contents of the two animals from which the bile had not been collected contained 32 / and 22 / of the administered dose.

Expiration study

Collection of expired CO_2 during the periods 0–½ ½–1 1–2, 2–4 and 4–6 hours after an intravenous dose of Lu 5-003 – ^{14}C showed the greatest rate of

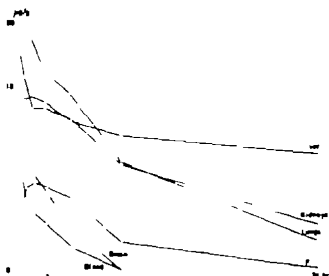


Fig 6. Distribution of radioactivity following administration of Lu 5-003 – ^{14}C. (n = 3).

Table 5

Amount of radioactivity in tissues expressed as µg Lu 5-003/g wet weight and % of dose. Intravenous administration (n = 3).

	5 min.		1 hour		4 hours		8 hours		24 hours	
	µg/g	%-dose	µg/g	%-dose	µg/g	%-dose	µg/g	%-dose	µg/g	%-dose
Blood*	0.94		0.42	–	0.18	–	0.07	–	0.03	–
Brain	1.2	0.4	1.1	0.4	0.3	0.1	0.1	0.0	< 0.02	–
Heart	12.6	1.0	2.6	0.2	0.7	0.0	0.3	0.0	0.1	0.0
Lungs	38.0	6.1	32.1	4.6	8.4	1.5	1.5	0.3	0.2	0.0
Liver	8.4	6.5	7.8	5.8	4.2	3.7	2.9	2.9	1.8	2.2
Kidneys	22.0	4.0	5.8	1.1	4.4	0.8	1.4	0.3	0.3	0.1
Spleen	4.0	0.3	9.6	0.6	2.3	0.2	1.0	0.1	0.2	0.0
Epididymal Fat	0.6	0.1	0.9	0.1	0.6	0.1	0.2	0.0	0.1	0.0
Gastro-intestinal Tract	3.7	9.0	12.1	27.4	11.9	45.9	11.0	34.7	3.0	9.7
Carcass	4.4	64.6	2.9	43.4	0.9	13.3	0.5	7.1	0.1	1.6
Tail		1.1		0.7		0.5		0.5		0.2
Total		93.1		84.3		66.1		45.9		13.8

*) µg/ml

expiration of radioactivity during the ½–1 hour period. A total of 0 04 / of the administered dose was excreted as $^{14}CO_2$ in 6 hours. This may indicate that the radioactive carbon dioxide originated from degradation of a radioactive impurity rather than from degradation of Lu 5-003

Distribution study

The distribution of radioactivity following intravenous injection of labelled Lu 5-003 is shown in table 5 and fig. 6. The largest concentration is found in the lungs during the first hours following administration. The kidneys, heart

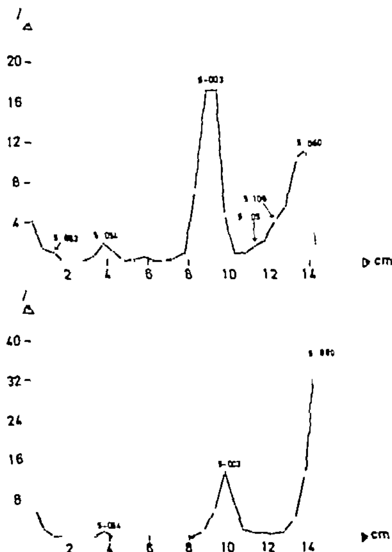


Fig 7 Radioactivity from chromatograms of basic extracts from brain in system 3 Upper: 5 min. after administration. Lower: 4 hours after administration.

and liver also contain great amounts, while the concentration in the brain is considerably smaller and only a little higher than the blood concentration. The concentration of radioactivity in the blood and the brain decreased rapidly with time giving a half-life of about 2 hours The decrease of radioactivity in the liver is considerably slower with a half-life of about 4 hours.

About 70–100 / of the radioactivity in the brain, the lungs and the kidneys was extractable at basic pH, while only 0–20 / could be extracted at acidic pH. From the liver the extractable amounts were 33–50 / and 2–13 / at basic and acidic pH, respectively Chromatography of the basic extracts from brain showed that 85–90 / of the radioactivity in the extracts occurred in the two amines Lu 5-003 and Lu 5-060 At the short times after administration (5 min. – 1 hour) Lu 5-003 was present in greater amounts than Lu 5-060 but later Lu 5-060 dominated (see fig. 7). Very small amounts of the sulphoxides of the amines were also seen. The amines also dominated in the basic extracts from the lungs and the kidneys with Lu 5-003 predominat ing at all times after administration. The sulphoxides were also seen but only in very small amounts. In the basic extracts from liver greater amounts of metabolites were seen than in extracts from other organs although the amines Lu 5-003 and Lu 5-060 still constituted the greatest part. The metabolites seen were the four sulphoxides Lu 5-052, Lu 5-054 Lu 5 105 and Lu 5-106 the propionic acid derivative Lu 5-085 and the sulphoxide of this compound, Lu 5-086.

Due to the low amount of radioactivity in the acid extracts from the organs these could not be chromatographed with a reasonable yield.

Discussion

Sulphoxidation is the most important pathway in the biotransformation of Lu 5-003 In addition to sulphoxidation, degradation of the side chain giving primary amines and propionic acid derivatives is seen. Further degradation of the side chain as suggested in human studies is not supported by the ex piration study with rats. However further degradation of the side chain may occur to a negligible extent. The side chain degradation is identical to the degradation of the corresponding phthalane Lu 3-010 (PLYM FORSHELL *et al* 1968). No hydroxylated metabolites are formed which is in contrast to the formation of large amounts of these from tricyclic thymoleptics, e. g. nortriptyline and desipramine.

No obvious differences are seen between the three species investigated. It is possible that the sulphoxides dominate somewhat more in humans than in the two animal species.

It is unexpected that elimination proceeds via the faeces to such a large extent even though the substance is intensively transformed to highly hydro-

philic metabolites. The study of biliary excretion indicates the presence of an enterohepatic circulation. It is also shown that the substances excreted in the faeces originate from biliary excretion rather than from excretion from glands or other sources.

The distribution of Lu 5-003 and its metabolites follows the well known pattern of psychotropic drugs, e. g. nortriptyline (McMahon *et al.* 1963), prothiadene (dosulepinum NFN) (Horešovský *et al.* 1967) and imipramine (Herrmann & Pulver 1960). Rather large amounts of the primary amine are found in all organs and this finding is similar to that made with Lu 3-010 (Plym Forshell *et al.* 1968). The amount of primary amine in the brain increases with time relative to parent substance.

The biological half life of Lu 5-003 and its metabolites in rats calculated from blood and brain concentrations is about 2 hours. This is close to that of imipramine (2½ hours) (Dingell *et al.* 1964) but considerably shorter than for Lu 3-010 (7 hours) (Plym Forshell *et al.* 1968) and desipramine (9 hours) (Dingell *et al.* 1964). The short half life of Lu 5-003 as compared to that of Lu 3-010 might be explained by the formation of the hydrophilic sulphoxide metabolites of Lu 5-003.

Acknowledgements

The authors wish to thank Dr. J. Ravn, State Hospital Middelfart, for supplying the human samples. Our thanks are also given to our laboratory staff for their skilful technical assistance.

REFERENCES

Dingell, J. V., F. Sulser & J. R. Gillette: Species differences in the metabolism of imipramin and desmethylimipramin (DMI). *J. Pharm. Exp. Ther.* 1964, 143, 14–22.

Herberg, R. J.: Determination of carbon-14 and tritium in blood and other whole tissues. *Anal. Chem.* 1960, 32, 1, 42–46.

Herrmann, B. & R. Pulver: Der Stoffwechsel des Psychopharmakons Tofranil. *Arch. int. Pharmacodyn.* 1960, 126, 454–469.

Horešovský, O., Z. Franc & P. Kraus: Biochemistry of Drugs XI. The metabolic fate of a new psychotropic drug 11-(3-dimethylaminopropylidene)-6,11-dihydrodibenz(b,e)-thiepine (prothiadene). *Biochem. Pharmacol.* 1967, 16, 2421–29.

McMahon, R. E., F. J. Marshall, H. N. Culp & W. M. Miller: The metabolism of nortriptyline – N Methyl ^{14}C in Rats. *Biochem. Pharmacol.* 1963, 12, 1207–1217.

Petersen, P. V., N. Lassen, T. Ammitzbøll, I. Møller Nielsen, M. Nymark, V. Pedersen & K. F. Franck: Further pharmacological studies of bicyclic thymoleptics. *Acta pharmacol. et toxicol.* in press.

Plym Forshell, G., P. Schaumann, V. Hansen, U. Dahl Larsen, A. Jørgensen & K. Fredricson Overø: Distribution and metabolism of 3,3-dimethyl-1-(3-methylaminopropyl)-1-phenyl-phthalane (Lu 3-010), a bicyclic compound with thymoleptic properties. *Acta pharmacol. et toxicol.* 1968, 26, 507–520.

Stahl, E.: *Dünnschicht-Chromatographie.* Springer Verlag, 1967.

From the Department of Pharmacology
University of Copenhagen, Denmark

Studies on the Subcutaneous Absorption in Mice VII Absorption of $^{3}H_2O$ and ^{14}C-sucrose from Non-buffered Solutions at Different pH Values

By

E. Secher Hansen

(Received August 7 1969)

Abstract The possible influence on the subcutaneous absorption process of the pH of the injected *unbuffered* solutions was examined in normal and oestradiol pretreated mice. Eighty µl of 0.9 % NaCl, pH 4.60, 5.70, and 8.90, containing $^{3}H_2O$ and ^{14}C-sucrose in tracer concentrations were injected. In the oestradiol pretreated mice, the clearance of the tracer substance was significantly faster at pH 4.60 than at pH 8.90. Addition of hyaluronidase to the solutions abolished these differences. The results point to the connective tissue ground substance as th medium capable of modifying the absorption process according to slight pH dif ferences of the injected solutions.

Key-words: Absorption – injection, subcutaneous.

Reports from this laboratory have dealt with the significance of the connective tissue ground substance in the subcutaneous absorption of solutions of different tonicity and of substances with different molecular weights (Secher Hansen, Langgård & Schou 1967b c & 1968 Secher Hansen 1968). The present study was designed to determine if small changes in the pH in the injected unbuffered solutions might be of significance to the absorption process.

Method

In lightly anaesthetized (halothane (fluothane ®)) mal albino mice (Leo, Stritt) two symmetrical areas were marked out on the depilated skin of the back (cf. Secher Hansen, Langgård & Schou 1967a). Eighty µl of 0.9 % NaCl containing about 0.3 µcl of $^{3}H_2O$ (New England Nuclear Corp. Boston, U.S.A.) or of ^{14}C-sucrose (The Radiochemical Center Amersham, England) was injected subcutaneously in the right side area to half of the animals in addition 40 I.u. of hyaluronidase (penetrase Leo ®). The solutions were adjusted, by means of 0.01 N-HCl and 0.01 N-NaOH, at the pH-values 4.60 5.70 and 8.90. Parallel experiments were carried out in mice pretreated with

oestradiol monobenzoate 10 μg in 100 μl arachis oil given subcutaneously six and four days before the experiment (cf. HVIDBERG, SZPORNY & LANGGÅRD 1963). The animals were decapitated 5 or 15 minutes after the subcutaneous injections, the two pieces of skin excised and their radioactivity contents determined by double tracer technique in a Packard Tri-Carb Liquid Scintillation Spectrometer model 3003 the differences were expressed in per cent of the injected dose ("residual radioactivity per cent").

Results

In the non-pretreated mice the changes in the pH of the injected solutions were without influence on the disappearance rates of the radioactive water and sucrose molecules (fig. 1) In the oestradiol treated mice, in addition to an overall slower absorption (cf. SECHER HANSEN 1968) sucrose- as well as water molecules disappeared significantly faster at pH 8.90 than at pH 4 60 (fig. 2). The differences were demonstrable after 5 minutes only With hyaluronidase added to the injected solution the two tracer substances disappeared at a rate independent of the pH values (tables 1 & 2)

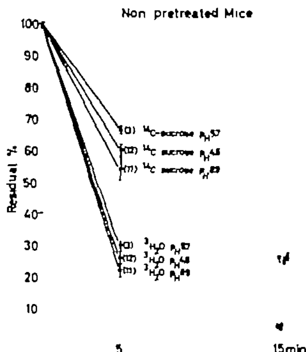


Fig. 1 Residual radioactivity per cent 5 and 15 minutes after subcutaneous injections of 80 μl of 0.9 % NaCl solutions with pH 4 60, 5 70 and 8.90 containing approximately 0.3 μci of ^3H_2O or ^{14}C-sucrose per dose (Non-pretreated mice). The vertical lines indicate the standard error of the mean. The figures in brackets indicate number of animal

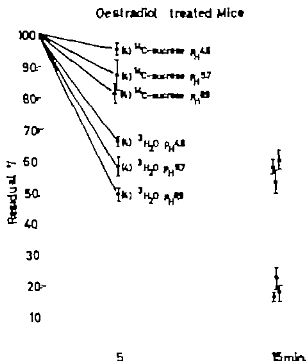


Fig. 2. Residual radioactivity per cent 5 and 15 minutes after subcutaneous injections of 80 µl of 0.9 % N Cl solutions with pH 4.60, 5.70 and 8.90 containing approximately 0.3 µci of ^3H_2O or ^{14}C-sucrose per dose (Oestradiol treated mice).

Table 1

Residual radioactivity per cent originating from ^3H_2O and ^{14}C-sucrose 5 and 15 minutes after the subcutaneous injections of 80 µl of 0.9 % NaCl containing approximately 0.3 µci of the tracer substances *plus 40 I. u. of hyaluronidase* (Non pretreated mice, n = number of animals).

pH	5 min.		15 min.	
	^3H_2O mean ± S. E. M.	^{14}C-sucrose mean ± S. E. M.	^3H_2O mean ± S. E. M.	^{14}C-sucrose mean ± S. E. M.
4.60	17 ± 1.3 (= 11)	38 ± 1.5 (n = 11)	2 ± 0.3 (n = 11)	9 ± 1.1 (n = 11)
5.70	18 ± 1.2* (n = 16)	40 ± 1.6 (n = 10)	1 ± 0.3 (= 4)	8 ± 0.9 (n = 4)
8.90	17 ± 2.0 (n = 11)	35 ± 2.4 (= 11)	2 ± 0.3 (n = 11)	9 ± 0.6 (n = 11)

) These two values have been published previously in Secher Hansen, Langgård & Schou (1967b).

7*

Table 2

Residual radioactivity per cent originating from $^{3}H_2O$ and ^{14}C-sucrose 5 and 15 minutes after the subcutaneous injections of 80 µl of 0.9 NaCl containing approximately 0.3 µci of the tracer substances *plus 40 I.U. hyaluronidase* (Oestradiol treated mice, n = number of animals).

pH	5 min.		15 min.	
	$^{3}H_2O$ mean ± S. E. M	^{14}C sucrose mean ± S. E. M	$^{3}H_2O$ mean ± S. E. M.	^{14}C-sucrose mean ± S. E. M.
4.60	34 ± 3.7 (n = 6)	54 ± 1.8 (n = 6)	8 ± 1.2 (n = 6)	26 ± 2.7 (n = 6)
5.70	44 ± 1.7 (n = 4)	63 ± 4.5 (n = 4)	7 ± 1.8 (n = 4)	22 ± 2.9 (n = 4)
8.90	35 ± 4.5 (n = 6)	52 ± 4.7 (n = 6)	9 ± 1.8 (n = 6)	22 ± 2.0 (n = 6)

Discussion

Only few investigations have been made on the influence of the pH of subcutaneously injected solutions on the rate of the absorption process. HVIDBERG (1958) found an increased spreading of solutions in the skin of mice when the pH was 5 or lower. The investigation was, however, carried out on killed mice, using solutions with considerable buffer capacity.

Previous investigations on the subcutaneous absorption of *unbuffered* solutions with different pH-values have seldom been made. LAURENT & PIETRUSZKIEWICZ (1961) could not demonstrate any relation *in vitro* between the pH and the sedimentation rate for larger molecules in a hyaluronic acid medium. MADISON & CHRISTIAN (1950) found by investigation *in vivo* that the absorption of sodium was uninfluenced by pH alterations between 2.5 and 10. However, below pH 2, a decreased and above pH 11 an increased rate of absorption was found. The results of the present study may be said to be in accordance with this, as a lower disappearance rate of water and sucrose molecules was observed at pH 4.60 than at pH 8.90. At these small degrees of acidosis and alkalosis, however, the changes were demonstrable only in the oestradiol pretreated animals, i.e. where the amounts of ground substance in the skin had been "artificially" doubled and the degree of polymerisation of the hyaluronic acid greatly increased (HVIDBERG, SZPORNY & LANGGÅRD 1963). This in turn points to the ground substance as the medium capable of modifying the absorption process when slight pH changes takes place. This was further substantiated by the fact that the differences in absorption rates were abolished when hyaluronidase was added to the solutions injected.

Acknowledgements

This study was supported by grants from the Danish National Association against Rheumatic Diseases, and was carried out in the Laboratory for Investigation of the Pharmacology of Connective Tissue. Mrs. L. Jacobsen gave perfect technical assistance.

REFERENCES

Hvidberg, E. Influence of hydrogen ion concentration on spread in subcutaneous connective tissue of the mouse. *Acta pharmacol et toxicol* 1958 14 400–406

Hvidberg, E., L. Szporny & H. Langgård. The composition of oedema fluid provoked in mice by oestradiol. *Acta pharmacol. et toxicol* 1963, 20 243–252.

Laurent, T C. & A. Pietruszkiewicz: Th effect of hyaluronic acid in the sedimentation rate of other substances. *Biochim. et Biophys. Acta* 1961, 49 258–264.

Madison, W L. & J B. Christian. The study of the absorption of sodium ions in the white rat using radioactive tracer techniques. *J Am. Pharmac Ass* 1950, 39 689–692.

Secher Hansen, E., H. Langgård & J Schou. Studies on the subcutaneous absorption in mice I A method for studying quantitatively the dynamics of subcutaneous absorption. *Acta pharmacol et toxicol* 1967a, 25, 162–168.

Secher Hansen, E., H. Langgård & J Schou. Studies on the subcutaneous absorption in mice II Influence of tonicity on the dynamics of subcutaneous absorption. *Acta pharmacol et toxicol* 1967b, 25 290–298.

Secher Hansen, E., H. Langgård & J Schou Studies on the subcutaneous absorption in mice III. Aseptic inflammation following subcutaneous injections. *Acta pharmacol. et toxicol* 1967c, 25 299–306.

Secher Hansen, E., H. Langgård & J Schou. Studies on the subcutaneous absorption in mice IV: Absorption of carbohydrates with different molecular weights from connective tissue. *Acta pharmacol et toxicol.* 1968, 26, 9–14

Secher Hansen, E.: Studies on the subcutaneous absorption in mice VI Absorption of mannitol, sucrose, inulin and albumin in normal and oestradiol treated animals. *Acta pharmacol. et toxicol* 1968, 26 316–324.

Acta pharmacol. et toxicol. 1970, 28 102–107

From the Department of Pharmacology
University of Copenhagen, Denmark

Studies on the Subcutaneous Absorption in Mice VIII Influence of Connective Tissue Ground Substance on the Absorption of Subcutaneously Injected Depots of Sodium and Chloride

By

E. Secher Hansen

(Received August 7 1969)

Abstract A standard volume of 80 µl of physiological saline or of pure water was injected subcutaneously in mice. In both cases the electrolyte composition of the depots was the same 15 minutes later at which time the injected volumes had not been significantly reduced. The sodium concentration in the depot corresponded to that of the plasma, while the chloride concentration corresponded to approximately 50 per cent of that of the plasma. The missing anions could probably be accounted for by the non-diffusible polyanions of the connective tissue ground substance. Radioactive sodium ions were cleared significantly more slowly than radioactive chloride ions, these in turn being significantly slower than the radioactive water molecules, after the subcutaneous injection of physiological saline or water depots containing the radioactive ions in tracer concentrations.

Key words: Absorption – injection, subcutaneous – connective tissue.

Previous experiments have shown that the disappearance of a subcutaneously injected volume and the exchange of water molecules and small ions between the depot and its surroundings are independent processes. While all the originally injected water molecules may have disappeared after a few minutes, the injected water-surplus at the same time may persist unchanged (Secher Hansen, Langgård & Schou 1967b). The present study was undertaken to clarify the movements of sodium and chloride in and out of a subcutaneously injected "depot" of physiological saline and of almost pure water

Method

Two symmetrical areas were marked out on th depilated skin of the back of albino male mice (cf. Secher Hansen, Langgård & Schou 1967a). Eighty µl of one of the following solutions were injected subcutaneously within the borders of the right side area.

1) Distilled water containing pproximately 1 µci of ^{24}NaCl per 80 µl.
2) Distilled water containing pproximately 0.5 µci of Na^{36}Cl per 80 µl.

The concentrations of sodium and chloride in these solutions were below 10 meq per litre.

3) 0.9 % saline containing approximately 1 µcl of $^{24}NaCl$ per 80 µl.
4) 0.9 % saline containing approximately 0.5 µcl of $Na^{36}Cl$, approximately 0.5 µcl of $^{3}H_2O$ and approximately 1 µcl of $^{24}NaCl$ per 80 µl.

Five or 15 minutes later the animals were decapitated and bled. The two pieces of skin (corium and subcutis) were excised and weighed. The differences in weight between the two samples were expressed in per cent of the volume injected ("residual volume per cent"). Their radioactivity contents due to ^{36}Cl and $^{3}H_2O$ were determined by the single or double tracer technique in Packard Tri-Carb Liquid Scintillation Spectrometer 3003, and the differences expressed in per cent of the injected radioactivity ("residual radioactivity per cent"). Their radioactivity contents due to ^{24}Na were determined in scintillation well-counter connected with an I. D. L. Scaler 1700 and th differences again expressed in per cent of the injected radioactivity. Subsequent chemical determinations of the sodium, potassium and chloride contents were carried out as described by LANGGÅRD, JENSEN-HOLM & HVIDBERG (1963). *The concentrations* of the electrolytes in the subcutaneous "depot" were calculated on the basis of the residual volumes and the excess of the ions on the injected sides.

Results

It is evident from fig. 1 that sodium and chloride ions are rapidly drawn into a subcutaneously injected depot of almost pure water. Fifteen minutes

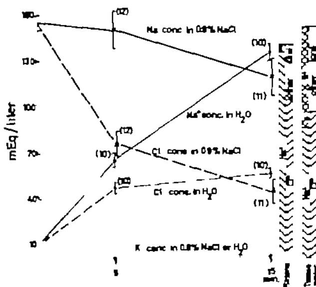


Fig. 1. *The concentration* of sodium, potassium and chloride in subcutaneously injected depots 5 and 15 minutes after the injection of 80 µl of 0.9 % NaCl or of 80 µl of distilled water. The concentration of anions and cations in th plasma and the tissue fluid is shown in columns. The vertical lines indicate the standard error of the mean. The figures in brackets indicate number of animals.

after the injection the sodium *concentration* in the depot has reached that of the plasma which is much higher than that of the normal tissue fluid. At this time the *concentration* of chloride in the depot has risen only to approximately *half of the concentrations of the plasma and tissue fluid.* – After subcutaneous injections of 0.9 / NaCl (155 meq per litre of sodium and 155 meq per litre of chloride) the *concentration* of sodium decreased to 120 meq. per litre during the initial 15 minutes of the absorption period. The chloride *concentration* in the depot had decreased at the same time to 45 meq per litre (fig. 1).

Radioactive sodium disappeared at a slower rate than radioactive chloride, which in turn disappeared at a slower rate than radioactive water molecules from subcutaneously injected "depots independent of the medium (fig. 2 & 3 values at 15 minutes: $P < 0\ 01$ fig. 3 values at 5 minutes, $P < 0\ 001$).

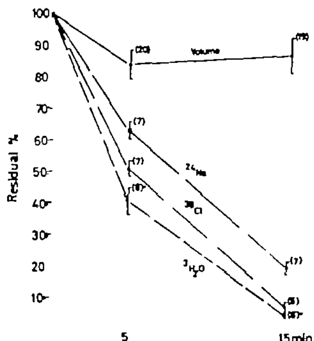


Fig. 2. *Residual volume per cent* and *residual radioactivity per cent* of ^{24}Na, ^{36}Cl and $^{3}H_2O$, 5 and 15 minutes after subcutaneous injections of 80 µl of distilled water containing approximately 1 µci of ^{24}NaCl or 0.5 µci of Na^{36}Cl or 3 µci of $^{3}H_2O$ per 80 µl. The concentration of sodium and chloride in the solutions was below 10 meq per litre. The vertical lines indicate the standard error of the mean. The figures in brackets indicate number of animals

The injected volumes were only insignificantly reduced 15 minutes after the injections (fig. 2 & 3)

Discussion

Since the structure of the connective tissue ground substance consists of a network of charged macromolecules the tissue possesses the capacity to *swell* i. e. to take up an excess of water as an integrate part of the tissue. The capacity for water binding seems to depend in particular on the concentration of hyaluronic acid (HVIDBERG 1962)

Due to the *polyanionic* properties of the macromolecules and at the pH existing in the tissue, the macromolecular network mentioned above carries a great negative net charge which is counteracted by (small) cations, i. e. hydrogen, sodium and potassium (LANGGÅRD 1965a). Thus the connective tissue contains an excess of small cations.

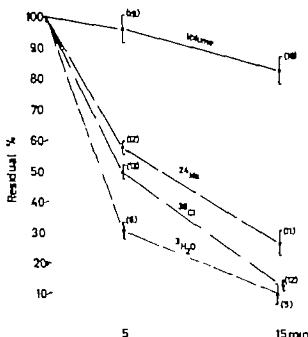


Fig. 3 *Residual volume per cent* and *residual radioactivity per cent* of ^{24}Na, ^{36}Cl and $^{3}H_2O$ 5 and 15 minutes after subcutaneous injections of 80 µl of 0.9 % NaCl containing approximately 1 µci of $^{24}NaCl$ or 0.5 µci of $Na^{36}Cl$ plus approximately 0.5 µci of $^{3}H_2O$ plus approximately 1 µci of $^{24}NaCl$ per 80 µl. The vertical lines indicate the standard error of the mean. The figures in brackets indicate number of animals.

The results of the present study seem to indicate that the negatively charged binding sites of the ground substance macromolecules are *available* for neutralization of an excess of sodium abruptly introduced into the tissue (fig. 1). Whether physiological saline or pure water is introduced subcutaneously the electrolyte composition of the depot in the course of 15 minutes, becomes the same. Thus concentration of sodium approximates to that of the plasma and the concentration of chloride to about 50 per cent of that of the plasma. This can hardly be a question of coincidence. Although bicarbonate ions may account for some of the missing anions the greater part of these must undoubtedly be accounted for by the polyanions of the tissue.

That the findings hitherto discussed are not due to differences in the diffusion rates of sodium and chloride is evident from fig. 2 and 3. The radioactive chloride ions were actually cleared at a significantly higher rate than the radioactive sodium ions. This discrepancy may be explained by differences in ion-size. As the sodium ion probably exists in the tissue in hydrated forms, the ion radius may be sufficiently increased to influence the rate of transport through the meshes of the ground substance (LANGGÅRD 1965a).

The explanation of the slower disappearance rate of chloride ions than of water molecules is obscure. The explanation may however be found in the possible existence of a slowly exchangeable fraction of the tissue chloride (LANGGÅRD 1965b).

Acknowledgements

This study was supported by grants from the Danish National Association against Rheumatic Diseases, and was carried out in the Laboratory for Investigation of the Pharmacology of Connective Tissue. The author would like to thank the Danish Atomic Energy Commision for putting the well-counter at his disposal

REFERENCES

Hvidberg, E. *Bindevævets vandindhold og grundsubstansens vandbinding.* (*The Water content of connective tissue and the water binding of the ground substance*). Thesis in Danish, with a summary in English. Andelsbogtrykkeriet, Odense, Denmark 1962.

Langgård, H., J. Jensen-Holm & E. Hvidberg: Connective tissue electrolytes during acidosis and alkalosis in normal and oestradiol-treated mice. *Acta pharmacol. et toxicol.* 1963, 20 121 130.

Langgård, H.. *Bindevævets elektrolytforhold.* (Connective tissue electrolytes). Thesis in Danish, with summary in English. Borgens Forlag, København, Denmark 1965a.

Langgård, H. Exchangeable water sodium and chloride in the skin of mice. *Acta physiol. Scand.* 1965b, 64 263–268.

Secher Hansen, E., H. Langgård & J. Schou: Studies on the subcutaneous absorption in mice I. A method for studying quantitatively the dynamics of subcutaneous absorption. *Acta pharmacol. et toxicol.* 1967a, 25 162 168.

Secher Hansen, E., H. Langgård & J. Schou. Studies on th subcutaneous absorption in mice II. Influence of tonicity on the dynamics of subcutaneous absorption. *Acta pharmacol. et toxicol.* 1967b, 25, 290–298.

Acta pharmacol. et toxicol. 1970 **28** 108–112.

From the Institute of Pharmacotherapeutics, University of Oslo,
Oslo, Norway

Experimentally Induced Cough in Man by Citric Acid Aerosol. An Evaluation of a Method

By

Finn O. Winther

(Received September 3 1969)

Abstract. Ten healthy males underwent three test periods with an interval of one hour. Each test period consisted of five inhalations of a citric acid aerosol. The number of coughs after each inhalation was registered. A placebo tablet was given immediately after the first test period. Statistical analysis of the results showed a small reduction in the number of coughs from the first to the second test period, but no significant reduction from the second to the third test period. The reduction might have been due to a placebo reaction. It is concluded that the method may be useful in evaluating an antitussive agent provided the investigation is carried out as a double blind test with placebo.

Key words: Cough – antitussive agents – respiratory system.

For several years an objective evaluation of antitussive agents has been the aim of scientists in various fields of medicine. Different methods have been described, but after a critical evaluation most of them can be rejected (GRAVENSTEIN *et al.* 1954). BICKERMANN *et al.* (1956) has described a method whereby a uniform cough response may be produced by inhalation of a citric acid aerosol. In a recent investigation, however SEVELIUS *et al.* (1965) found considerable adaptation to inhalations of citric acid aerosol. No explanation for the discrepancy was given.

The purpose of this investigation was to see whether an adaptation to citric acid aerosol inhalation is present when a subject is repeatedly stimulated with the same concentration, and also whether a placebo may have any influence on the individual reaction to the inhalations.

Material and methods

Ten men, between 20 and 50 years of age, were tested. None of these had any disease of the respiratory tract and none were heavy smokers (less than 10 cigarettes a day).

Table 1

The distribution of the test subjects according to the concentration of the citric acid solution.

	5 %	10 %	15 %	20 %	25 %
Number of persons	3	2	3	1	1

A block diagram of the apparatus used for nebulizing the citric acid solution has been given in a previous paper (WINTHER 1966), which also reports the results of an evaluation of an antitussive agent (meprotixol).

The citric acid solution was nebulized in an air flow of 8 l/min. ("Atomisor" La Diffusion Technique, Saint-Etienne, France. Particle size according to the firm 1 to 5 micron).

Each subject had a training period. During this period the concentration of nebulized citric acid solution which could produce between 3 and 6 coughs after one single inhalation was determined. The concentration ranged from 5 % to 25 %. The distribution is given in table 1. Initially the reaction was very inconstant, but after 5 to 8 inspirations the number of coughs became stabilized at an apparently constant level, at which we started the registrations. There were three test periods with an interval of one hour. Each test period was preceded by about 5 inhalations which were not taken into account.

Placebo tablets were administered immediately after the first test period. The subjects tested were informed that they would be given either an antitussive tablet or a placebo tablet. Each of the three test periods consisted of 5 inhalations with at least a 3 min. interval. After each inhalation the number of coughs was counted. As soon as the cough had stopped, the subject tested was given a few sips of lukewarm water so as to clear the mouth and the throat of citric acid.

Table 2a.

Number of coughs after inspiration of citric acid aerosol.

	1st test period						2nd test period						3rd test period					
Inspiration no.	1	2	3	4	5	Sum	1	2	3	4	5	Sum	1	2	3	4	5	Sum
							Number of coughs											
Subject no. 1	3	3	4	4	3	17	3	3	3	4	3	16	4	4	3	3	4	18
2.	4	2	3	2	2	13	4	1	3	2	2	12	4	2	2	3	2	13
3	4	3	3	3	3	16	3	3	3	2	3	14	3	3	2	2	2	12
4.	3	3	3	3	3	15	2	3	2	2	3	12	3	2	3	2	2	12
5.	5	4	5	4	4	22	4	4	3	2	3	16	4	3	3	3	2	15
6.	4	4	4	3	4	19	4	3	4	4	3	18	5	3	4	4	4	20
7	3	1	2	2	2	10	4	4	3	3	3	17	4	2	2	2	3	14
8	3	4	4	3	4	18	5	4	4	4	3	20	4	3	3	2	2	14
9.	4	3	3	4	3	17	4	4	2	3	2	15	4	3	2	2	3	14
10.	6	5	5	5	6	27	6	4	4	6	4	24	6	4	4	5	4	23
Sum	39	32	36	33	34	174	39	33	31	32	29	164	41	30	28	28	28	155

Table 2b

Total number of coughs after inspiration of citric acid aerosol in three test periods.

	Inspiration no. 1	2	3	4	5
	Number of coughs				
1.	10	10	10	11	10
2.	12	5	8	7	6
3	10	9	8	7	8
4	8	8	8	7	8
5	13	11	11	9	9
6.	13	10	12	11	11
7	11	7	7	7	8
8.	12	11	11	9	9
9	12	10	7	9	8
10.	18	13	13	16	14
Sum	119	95	95	93	91

Table 3

Analysis of variance and F-values.

	Degrees of freedom	Sum of squares	Mean squares
Among persons ..	9	70.7	7.9
First test period versus second and third	1	2.8	2.8
Second test period versus third	1	0.8	0.8
Error	18	17.2	0.96
First inspiration versus four other inspirations ..	1	17.3	17.3
Remaining degrees of freedom among inspirations ..	3	0.4	0.1
Interaction inspirations x subjects	36	12.0	0.33
Error	80	29.5	0.37
	149	150.7	

First test period versus second and third test period $F = \frac{2.8}{0.96} = 2.9$

Second test period versus third test $F = \frac{0.8}{0.96} < 1$

First inspiration test versus the other four tests $F = \frac{17.3}{0.37} = 46.8$

Among the four other inspirations $F = \frac{0.1}{0.37} < 1$

Results

The results are shown in tables 2a and b

There is a moderate reduction in the number of coughs from one test period to the next, and a marked fall within each test period. The latter fall is, however only seen between the first and the second inspiration.

Table 3 shows an analysis of variance.

The F-value for the first versus second and third test period is 2.9 corresponding to P ≈ 0.10 i.e. the probability of getting a difference as great or greater purely by chance is about 10 %.

In contrast the difference between the second and the third test period is clearly not-significant. The F value for first versus the four other inspirations is 46.8, corresponding to $P < 0.001$ i.e. a difference of this magnitude is extremely unlikely to occur by chance. The differences among the other four inspirations are not-significant.

Discussion

In agreement with BICKERMANN (1956) we found that after the initial adaptation during the training period, further adaptation to citric acid aerosol appeared to be small taking into account the whole test periods. The decrease between the first and the second test period might be ascribed to a placebo reaction. Within each test period, however a clearly significant reduction from the first inspiration to the following four inspirations occurred. We regard this reduction to be due to an adaptation similar to that seen in the training period.

The discrepancy between our observations and those found by SEVELIUS *et al.* (1965) is difficult to explain, but may be due to a small difference in the method. SEVELIUS (1965) used a concentration of citric acid solution that consistently induced the greatest number of coughs. We found it of great importance to find the weakest solution that produced between 3 and 6 coughs as early as possible in the training period. We assumed that if we used a stronger solution a greater number of coughs would be induced during the first inspirations, and that the adaptation would be subsequently more marked than with a weaker solution. We have not carried out any experiments to investigate this theory.

We feel that the method used in our experiments, together with studies in patients with cough from chronic broncho-pulmonary diseases, may be helpful in evaluating an antitussive agent. It is however essential that the investigation should be performed as a double blind test with placebo.

Acknowledgement

For valuable assistance in statistical evaluation I should like to thank Knut B Westlund, M. D the Life Insurance Companies' Institute for Medical Statistics at the Oslo City Hospitals.

REFERENCES

Bickermann, H. A., B. M. Cohen & M. German The cough response of normal human subjects stimulated experimentally by citric acid aerosol. Alterations produced by antitussive agents. Part I Methodology *Amer J med. Sci.* 1956, 232, 57–60.

Gravenstein, J S., R. A. Devloo & J K. Beecher Effects of antitussive agents on experimental and pathological cough in man. *J appl. Physiol.* 1954 7 119–139

Sevelius, H., P D Lester & J F Colmore Objective evaluation of antitussive agents. *Clin. Pharmacol. Therap.* 1965, 6, 146–151.

Winther F Ø Antitussive and side effects of meprotixol on experimentally induced coughing in man. *Acta pharmacol. et toxicol.* 1966 24, 134–138.

Acta pharmacol et toxicol. 1970 28, 113–123

From the Department of Biochemistry A,
University f Copenhagen, Denmark

The Influence of 2,4-Dinitrophenol on Metabolic Changes Caused by Ethanol in the Perfused Rat Liver

By

P B. Vendsborg and P Schambye*

(Received July 24 1969)

Abstract The experiments were undertaken in order to investigate the mechanism by which ethanol inhibits the tricarboxylic acid cycle. Isolated livers were perfused with human erythrocytes suspended in an artificial plasma Ethanol was added 45 min. after the start of the perfusions. In some experiments dinitrophenol was added after ethanol. The O_2 consumption in the perfused rat liver was unaltered by the addition of ethanol to the medium, whereas the CO_2 production fell considerably resulting in decrease in the RQ from 0.8 to 0.4 The addition of ethanol caused an increase in both lactate/pyruvate and hydroxybutyrate/acetoacetate ratios in the perfusion medium. When DNP was added to the perfusion medium, half an hour after the addition of ethanol, both the O_2 uptake and CO_2 production increased. The RQ increased from 0.4 to 0.6. The lactate/pyruvate ratio was increased by the addition of DNP because the amount of lactate liberated from the liver was increased, while the pyruvate concentration was almost unchanged. The addition of DNP caused a fall in the hydroxybutyrate/acetoacetate ratio from 2.2 to 0.6, and a net disappearance f ketone bodies was observed. An opposite change in the extra- and intramitochondrial NADH/NAD ratio was observed, favouring the hypothesis that they are really independent of each other A correlation between the intramitochondrial NADH/NAD ratio and the tricarboxylic acid cycle inhibition was demonstrated.

Key-words: Alcohol, ethyl metabolism krebs cycle dinitrophenols.

Ethanol oxidation is known to cause considerable changes in the liver metabolism. Normally the liver oxidizes fatty acids (FRITZ 1961) and only to a minor degree carbohydrates (MAJCHROWICZ & QUASTEL 1963 PESCH & TOPPER 1963) The CO_2 is therefore almost exclusively derived from the tricarboxylic acid cycle.

Present address: Department of Biochemistry University of Odense, Denmark.

When ethanol is oxidized by the liver this involves 75 % or more of its oxidizing capacity (LUNDQUIST *et al.* 1962, FORSANDER 1966, LELOIR & MUNOZ 1938). Ethanol is oxidized to acetate and most of it is not degraded further (LELOIR *et al.* 1938, FORSANDER & RÄIHÄ 1960). This partial oxidation of ethanol results in very low CO_2-production and RQ-value for the ethanol oxidation by the liver as shown by LUNDSGAARD (1938) and FORSANDER, RÄIHÄ, SALASPURO & MÄENPÄÄ (1965).

The constant O_2-uptake and low CO_2-production indicate that the normally working TCA-cycle is blocked. The mechanism by which this block is established is not known.

The redox state of the liver changes towards a more reduced condition when ethanol is oxidized (SMITH & NEWMAN 1959, RÄIHÄ & OURA 1962). It has been shown that the lactate/pyruvate ratio in the liver is an indication of the extramitochondrial NADH/NAD ratio (HOLZER, SCHULTZ & LYNEN 1956, BÜCHER & KLINGENBERG 1958). This is also reflected in the blood (HUCKABEE 1958) and the perfusion medium used in rat liver perfusions (SCHIMASSEK 1963) by the same substrate pair. In the same way the hydroxybutyrate/acetoacetate ratio can be taken as a measure of the intramitochondrial NADH/NAD ratio (KLINGENBERG & VON HÄFEN 1963, BORST 1963, WILLIAMSON, LUND & KREBS 1967).

The shift towards a more reduced state of the NADH/NAD system when ethanol is oxidized has been suggested as the cause of the TCA-cycle inhibition. Experiments have been carried out in order to correlate the redox state intra-and extramitochondrially with the function of the TCA-cycle. The lactate/pyruvate and hydroxybutyrate/acetoacetate ratios were used as parameters for the extra and intramitochondrial NADH/NAD ratios respectively. The function of the TCA-cycle was registered by means of the CO_2-production. DNP was given to alter selectively or at least predominantly the intramitochondrial redox state. A preliminary account of this work has been published (VENDSBORG & SCHAMBYE 1968).

Materials and methods

The chemicals were of the analytical grade. Enzymes for enzymatic determination were purchased from Boehringer Mannheim. The substrate determinations on the perfusion medium were performed by the following methods. Lactate (HOHORST 1962), pyruvate (BÜCHER, CZOK, LAMPRECHT & LATZKO 1962), hydroxybutyrate and acetoacetate (BERGMEYER & BERNT 1965). The addition of DNP did not influence the estimation and recovery of either the hydroxybutyrate or acetoacetate. DNP did not stimulate the conversion of acetoacetate to acetone in the perfusion medium.

The O_2-uptake and CO_2-production were measured by estimation of the total O_2 and CO_2 content in the medium entering and leaving the perfused liver. The total O_2 content was estimated according to the method of LAVER, MURPHY, SEIFEN & RADFORD

(1965) and the total CO_2 content by the method of SEVERINGHAUS (1962). The samples were taken up in glass syringes avoiding contact with air. After mixing the reagents used for driving out the gases, the pCO_2 and pO_2 were measured with a Radiometer pCO_2 electrode type E 5036 and pO_2 electrode type E 5046 respectively.

The operation and perfusion were performed according to the technique of HEMS (HEMS, ROSS, BERRY & KREBS 1966) with minor alterations. Male Wistar rats were used weighing 250–300 g and fed ad libitum on laboratory chow.

For the cannulation of the portal vein a needle connected with an infusion apparatus was used and an infusion with oxygenated perfusion medium (5 ml/min.) was started at once in order to reduce the anoxic period to a few seconds. The inferior caval vein was cut below the right renal vein in order to allow the infused blood to flow out freely while the thorax was opened and the operation finished. The caval and portal catheters were provided with threeway stopcocks at the entrance and outflow points. This allowed for the estimation of samples for CO_2 and O_2 to be taken without gas exchange with the surrounding air through the tubings. The samples for substrate estimations were taken through the stopcock on the caval catheter. As well as the fan heater there was a thermostate controlled plate heater under the reservoir to keep the perfusion medium at a constant temperature of 36°. The circulating pump was a peristaltic pump model 600-1200 from Harward apparatus Co. Inc., Dover Mass., U.S.A.

The composition of the perfusion medium was: 137 mM NaCl, 2.68 mM KCl, 1.80 mM $CaCl_2$, 0.49 mM $MgCl_2$, 10 mM phosphate, 2.6 % (w/v) bovine serum albumin powder, fraction V (Armour Pharmaceutical Co. LTD. Eastbourne, Sussex) and 2.5 %

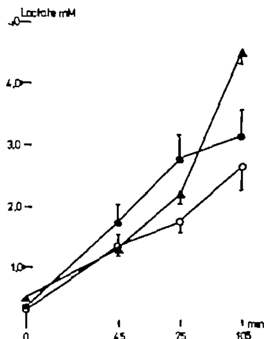


Fig. 1. Lactate concentrations in the perfusion medium. Experimental details are given in the text. ○ Control experiments with saline added (7 experiments). ●: Experiments with ethanol added at 45 min. (7 experiments). ▲ Experiments with ethanol added at 45 min. and DNP at 75 min. (6 experiments). The bars represent S. E. M.

(w/v) haemoglobin as human red cells washed twice with saline. The pH was adjusted to 7.3–7.4

The pressure in the portal vein was 8–13 cm H_2O and the flow around 20 ml/min. The caval catheter outlet was kept 15 cm below the liver. The livers weighed 10–12 g. The medium (150 ml) was oxygenated with 100 % oxygen.

After a 45 min. equilibration, period samples of the perfusion medium were taken and 3 ml ethanol (1 M) was added, 30 min. later samples were again taken and 1 ml ethanol (1 M) and 4 ml DNP (25 mM) were added. After another 30 min. the last samples were taken. The perfusion volume was kept constant by addition of more perfusion medium approximately 5 ml after each sampling. In the control experiments with no ethanol or DNP saline was added in the same amounts. Before the perfusion was started, lactate, pyruvate, hydroxybutyrate and acetoacetate concentrations were estimated in the perfusion medium.

Results

Changes in lactate and pyruvate concentrations

During the perfusion period there was a steady rise in the lactate and pyruvate concentrations in the control perfusion which did not level off

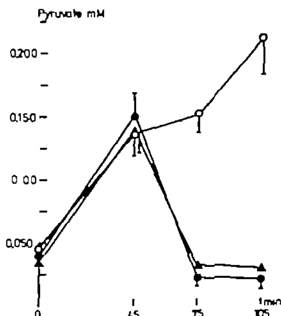


Fig. 2. Pyruvate concentrations in the perfusion medium. Experimental details are given in the text. ○ Control experiments with saline added (7 experiments). ● Experiments with ethanol added at 45 min. (7 experiments). ▲ Experiments with ethanol added at 45 min. and DNP at 75 min. (6 experiments). The bars represent S. E. M. (Missing bars too small to be drawn)

Table 1

Effect of ethanol and ethanol + DNP on lactate/pyruvate and hydroxybutyrate/acetoacetate ratios in the perfusion medium. Experimental details are given in the text. In the experiments in which additions were made ethanol was added after 45 min. and DNP after 75 min.

Sampling time min.	Lactate/pyruvate ratio			Hydroxybutyrate/acetoacetate ratio		
	Control	Ethanol added	Ethanol + DNP added	Control	Ethanol added	Ethanol + DNP dded
45	10	12	10	0.81	1.32	0.87
75	11	126	68	0.91	2.91	2.17
105	12	150	152	0.93	2.54	0.59

during the period (figs. 1 and 2) This is in contrast to the finding of SCHIMASSEK (1963) who obtained a constant level within 30 min. and of FORSANDER, RÄIHÄ, SALASPURO & MÄENPÄÄ (1965) who within 60 min, obtained a maximal value which decreased in the following two hours.

Table 2

Effect of ethanol and ethanol + DNP on the total concentration of lactate + pyruvate and hydroxybutyrate + acetoacetate in the perfusion medium. Experimental details are given in the text. Additions are made as in table 1 Th concentrations are given as means ± S. E. M. with the number of determinations in brackets

Sampling time min.	Lactate + pyruvate concentrations in mM		
	Control	Ethanol added	Ethanol + DNP added
0	0.33 ± 0.042 (14)	0.36 ± 0.027 (14)	0.54 ± 0.033 (12)
45	1.49 ± 0.14 (14)	1.90 ± 0.24 (14)	1.47 ± 0.08 (12)
75	1.89 ± 0.13 (14)	2.79 ± 0.35 (14)	2.22 ± 0.11 (12)
105	2.86 ± 0.36 (14)	3.18 ± 0.38 (14)	4.52 ± 0.12 (12)

Sampling time min	Hydroxybutyrat + acetoacetate concentrations in mM		
	Control	Ethanol added	Ethanol + DNP added
0	0.038 ± 0.007 (12)	0.026 ± 0.005 (8)	0.083 ± 0.013 (12)
45	0.70 ± 0.053 (12)	0.65 ± 0.072 (8)	0.55 ± 0.026 (12)
75	1.11 ± 0.090 (12)	0.84 ± 0.075 (8)	0.72 ± 0.044 (12)
105	1.40 ± 0.132 (12)	1.03 ± 0.103 (8)	0.60 ± 0.036 (12)

The addition of ethanol caused a slight increase in lactate concentration whereas the pyruvate concentration decreased approximately 10 times.

After the addition of DNP there was a significant ($P < 0.001$) rise in lactate concentration from 2.20 to 4.49 mM which was not seen in the control experiments with alcohol alone. The pyruvate concentration was unaffected by DNP addition.

The ratio lactate/pyruvate (table 1) which remained constant in the control experiments without ethanol at a value around 10 increased with the addition of ethanol by approximately 10 times in 30 min. In the control with alcohol it continued to rise slowly in the next 30 min., whereas the addition of DNP seemed to cause a further increase in the lactate/pyruvate ratio which was due to the more pronounced rise in the lactate concentration.

Compared to the normal values the total amount of lactate and pyruvate (table 2) in the medium increased only slightly more under the influence of ethanol, whereas the addition of DNP gave a significant rise.

Hydroxybutyrate and acetoacetate concentration.

The concentration of hydroxybutyrate increased throughout the control experiments without addition of ethanol or DNP and there was no significant difference between the experiments with or without ethanol. The addition of

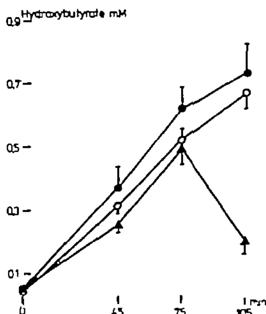


Fig. 3. Hydroxybutyrate concentrations in the perfusion medium. Experimental details are given in the text. ○ Control experiments with saline added (6 experiments). ● Experiments with ethanol added at 45 min. (4 experiments). ▲ Experiments with ethanol added at 45 min. and DNP at 75 min. (6 experiments). The bars represent S.E.M.

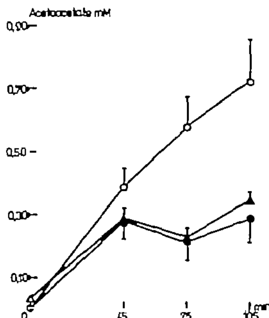


Fig. 4 Acetoacetate concentrations in the perfusion medium. Experimental details are given in the text. ○ Control experiments with saline added (6 experiments). ● Experiments with ethanol added at 45 min. (4 experiments). ▲ Experiments with ethanol added at 45 min. and DNP at 75 min. (6 experiments). The bars represent S. E. M.

DNP caused a very steep decrease in the hydroxybutyrate concentration and brought it to a level which was as low as that before the addition of ethanol (fig. 3).

The concentration of acetoacetate also rose steadily throughout the control experiments (without additions), but the addition of ethanol caused a large decrease in the concentration (fig. 4) In the next 30 min. there was a slight but not significant rise in the acetoacetate concentration in the experiments with only ethanol added ($P < 0.4$), whereas the addition of DNP caused a significant rise from 0.23 mM to 0.35 mM ($P < 0.001$).

In the normal perfusions without any additions the hydroxybutyrate/acetoacetate ratio (table 1) was nearly constant. Addition of ethanol caused a nearly 3 fold increase which was abolished completely by the subsequent addition of DNP

In the normal perfusion there is a steady output of ketone bodies (table 2) from the liver whereas the addition of ethanol slows down this release. The addition of DNP causes a fall in total ketone body concentration which is due to an uptake of ketones from the medium. The total uptake in the 30 minutes period is approximately 25 μmoles or nearly 25 / of the total amount present.

The addition of ethanol caused a slight increase in lactate concentration whereas the pyruvate concentration decreased approximately 10 times.

After the addition of DNP there was a significant ($P < 0.001$) rise in lactate concentration from 2.20 to 4.49 mM which was not seen in the control experiments with alcohol alone. The pyruvate concentration was unaffected by DNP addition.

The ratio lactate/pyruvate (table 1) which remained constant in the control experiments without ethanol at a value around 10 increased with the addition of ethanol by approximately 10 times in 30 min. In the control with alcohol it continued to rise slowly in the next 30 min., whereas the addition of DNP seemed to cause a further increase in the lactate/pyruvate ratio which was due to the more pronounced rise in the lactate concentration.

Compared to the normal values the total amount of lactate and pyruvate (table 2) in the medium increased only slightly more under the influence of ethanol whereas the addition of DNP gave a significant rise.

Hydroxybutyrate and acetoacetate concentration

The concentration of hydroxybutyrate increased throughout the control experiments without addition of ethanol or DNP and there was no significant difference between the experiments with or without ethanol. The addition of

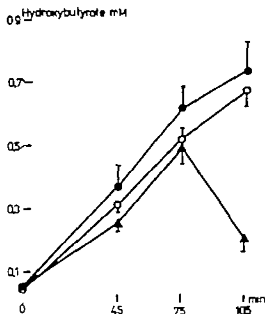


Fig. 3 Hydroxybutyrate concentrations in the perfusion medium. Experimental details are given in the text. ○ Control experiments with saline added (6 experiments). ●: Experiments with ethanol added at 45 min. (4 experiments). ▲ Experiments with ethanol added at 45 min. and DNP at 75 min. (6 experiments). The bars represent S. E. M.

lactate concentration, and as seen in table 2 there is no change in total lactate plus pyruvate. This indicates a shift in the extramitochondrial redox state and not an effect on glycolysis or gluconeogenesis.

The addition of DNP causes a significant increase in total lactate plus pyruvate due to a steep increase in lactate concentration. This could be caused by an increased availability of ADP for the glycolysis, i. e. an inhibition of the Pasteur effect.

DNP does not significantly influence the resulting lactate/pyruvate ratio indicating that it has no direct effect on the extramitochondrial redox state.

The addition of alcohol does not increase the amount of ketones liberated to the perfusion medium. It has previously been shown by others (WARMING-LARSEN 1949 FORSANDER *et al.* 1965 LUNDQUIST *et al* 1962) that alcohol has no ketogenic effect. In the present experiments there was a tendency to decrease total ketone body formation, since the shift in hydroxybutyrate/acetoacetate ratio produced by alcohol is brought about by reducing the acetoacetate concentration with no corresponding increase in hydroxybutyrate concentration, resulting in a decreased ketone body level. The explanation might be that the increase in NADH/NAD ratio produced by ethanol sti mulates lipogenesis or inhibits lipolysis at a step prior to the formation of acetyl-coA.

When DNP is added the need for extra oxidizable substrates is partly met by hydroxybutyrate, the concentration of which decreases considerably The concentration of acetoacetate rises significantly but not to such an extent that the hydroxybutyrate concentration decreases. The resulting total fall in ketone bodies is of approximately the same magnitude as reported by WEINHOUSE & MILLINGTON (1951) in slice experiments. If one assumes that the hydroxybutyrate is metabolized to CO_2 and H_2O its combustion represents around $^1/_6$ of the total O_2 uptake in the period.

O_2-uptake was found to be very sensitive to temperature and the tempera ture of 36 used in the experiments may possibly explain the slightly lower rate found in our experiments than in those reported by others (FORSANDER *et al.* 1965 SCHIMASSEK 1962) The decrease in RQ when ethanol is oxidized, is seen very clearly in table 3 The rise in RQ caused by DNP indicates that the inhibition of the TCA-cycle is released.

After DNP is added, a shift is seen in the opposite direction of the lactate/pyruvate ratio and hydroxybutyrate/acetoacetate ratio which confirm the belief in two entirely different nicotinamide adenine dinucleotide pools for the extra-and intramitochondrial dehydrogenases.

The ethanol which is oxidized extramitochondrially increases the extrami tochondrial NADH/NAD ratio which by some transport mechanism increases the intramitochondrial NADH/NAD ratio, and at the same time decreases the CO_2-production

The possibility of achieving an extensive oxidation of the intramitochondrial NADH, with a relatively small increase in oxydation rate suggests that the transporting mechanism for extramitochondrial reduction equivalents normally works at a maximal or nearly maximal rate.

Acknowledgement

This work was supported by grants from NOVO Industri A/S and from Statens almindelige Videnskabsfond. The skilled technical assistance of Miss L. Engbæk is gratefully acknowledged.

REFERENCES

Bergmeyer, H. U. & E. Bernt: Enzymatische Bestimmung von Keton Körpern im Blut. *Enzymol. biol. clin.* 1965, 5, 65–76.

Borst, P.: In: *Funktionelle und Morphologische Organization der Zelle* 1963. Ed. by Karlson, P. Berlin, Springer Verlag, p. 137.

Bücher, T., R. Czok, W. Lamprecht & E. Latzko: In. *Methoden der enzymatischen Analyse* 1962. Ed. by Bergmeyer, H. U. Weinheim/Bergstr. Verlag Chemie, p. 253.

Bücher, T. & M. Klingenberg: Wege des Wasserstoffs in der lebendigen Organisation. *Angew. Chem.* 1958, 70, 552–570.

Pondal, E. & C. D. Kochakian: *In vitro* effect of ethyl alcohol on respiration of rat liver and kidney slices. *Proc. Soc. exp. Biol.* N. Y. 1951, 77, 823–824.

Forsander, O. A.: Influence of the metabolism of ethanol on the lactate/pyruvate ratio of rat liver slices. *Biochem. J.* 1966, 98, 244–247.

Forsander, O. A. & N. C. R. Räihä: Metabolites produced in the liver during alcohol oxidation. *J. biol. Chem.* 1960, 235, 34–36.

Forsander, O. A., N. Räihä, M. Salaspuro & P. Mäenpää: Influence of ethanol on the liver metabolism of fed and starved rats. *Biochem. J.* 1965, 94, 259–265.

Fritz, I. B.: Factors influencing the rates of long-chain fatty acid oxidation and synthesis in mammalian systems. *Physiol. Rev.* 1961, 41, 52–129.

Hems, R., B. D. Ross, M. N. Berry & H. A. Krebs: Gluconeogenesis in the perfused rat liver. *Biochem. J.* 1966, 101, 284–292.

Hohorst, H. J.: In. *Methoden der enzymatischen Analyse*. Ed. by Bergmeyer, H. U. Weinheim/Bergstr. Verlag Chemie, p. 215.

Holzer, H., G. Schultz & F. Lynen: Bestimmung des Quotienten DPNH/DPN in lebenden Hefezellen durch Analyse stationärer Alkohol und Acetaldehyd-Konzentrationen. *Biochem. Z.* 1956, 328, 252–263.

Huckabee, W. E.: Relationships of pyruvate and lactate during anaerobic metabolism. *J. clin. Invest.* 1958, 37, 244–254.

Klingenberg, M. & H. von Häfen: Wege des Wasserstoffs in Mitochondrien. *Biochem. Z.* 1963, 337, 120–145.

Laver, M. B., A. J. Murphy, A. Seifen & E. P. Radford, Jr.: Blood O_2 content measurements using the oxygen electrode. *J. Appl. Physiol.* 1965, 20, 1063–1069.

Leloir, L. F. & J. M. Munoz: Ethyl alcohol metabolism in animal tissues. *Biochem. J.* 1938, 32, 299–307.

Lundquist, F N Tygstrup, K. Winkler K. Mellemgård & S. Munck Petersen Ethanol metabolism and production of free acetate in the human liver *J clin Invest* 1962, 41, 955–960.

Lundsgaard, E. Alcohol oxidation as a function of the liver *C. R. Lab Carlsberg Ser chim.* 1938, 22, 333–337

Majchrowicz, E. & J H. Quastel Effects of aliphatic alcohols on th metabolism of glucose and fructose in rat liver slices. *Canad J Biochem. Physiol* 1963, 41 793–803

Peach, L. A. & Y L Topper In. *Th Liver* Ed. by Rouiller Ch., New York, Academic Press Inc p. 610.

Räihä, N C. R. & E Oura: Effect of ethanol oxidation on levels of pyridine nucleotides in liver and yeast. *Proc Soc exp Biol N Y* 1962, 109 908–910.

Schimassek, H. Der Einfluss der Leber auf den extracellulären Redox-Quotienten Lactat/Pyruvat. *Biochem. Z.* 1963 336 468–473

Severinghaus, J W Electrodes for blood and gas P_{CO_2}, P_{O_2} and blood pH *Acta Anesth Scand. S ppl.* 1962, 11, 207–220.

Smith, M. E. & H. W Newman: The rate of ethanol metabolism in fed and fasting animals. *J biol. Chem.* 1959, 234 1544–1549

Warming-Larsen, A. The influence of alcohol on ketone metabolism. *Acta Med Scand.* 132, 458–465

Wenhouse, S. & R. Millington. A study of acetoacetate "turnover" in rat liver slices *in vitro. J biol Chem.* 1951 193, 1–10

Vendsborg, P B. & P Schambye: The influence of 2,4-dinitrophenol on th metabolic changes caused by ethanol in the perfused rat liver *Abstr 5th Meet. Fed. Europ Biochem. Soc.* 1968, FC 459 115

Williamson, D H., P Lund & H. A. Krebs: The redox state of free nicotinamide adenine dinucleotide in the cytoplasm and mitochondria of rat liver *Biochem. J* 1967 103 514–527

Acta pharmacol et toxicol 1970 28, 124–137

From the Department of Pharmacology Institute of Pharmacy
University of Oslo Norway

Determination of Factors of the Kinin System in Rat Plasma

By

Kjell Briseid, Olav Kjell Dyrud and Svein Öie
(Received September 5 1969)

Abstract. Methods were developed for determining the kininogen fractions, kininase and prekallikrein. The plasma prekallikrein was activated by 20 % (v/v) acetone for about 17 hours (20– 4). Urine kallikrein was prepared by dialysis of urine against running tap water for about 24 hours. Kininase activity was eliminated in plasma, plasma kallikrein and urine kallikrein by incubation at 37 with EDTA 2Na (1.0×10^{-2} M) for about 24 hours. Kinin assays were carried out on the isolated rat uterus. Released kinin was calculated as µg bradykinin/ml plasma. The total kininogen, whether determined by activation with acetone (16 % /v for not less than 5 hours) and subsequent incubation with plasma kallikrein, by incubation with plasma kallikrein and then urine kallikrein, or by incubation with acetone (20 % v/v for 17 hours) and subsequent evaporation of the acetone, was found to be the same 2.0 µg/ml plasma as an average value of 7 plasma batches corresponding to a total of 90 rats (S. D = 0.09). The average values of kinin released by incubation with plasma kallikrein and by urine kallikrein were 1.5 µg/ml and 1.4 µg/ml plasma respectively with S. D values of 0.11 and 0.06 respectively The procedures for kininase and for prekallikrein determinations corresponded closely to previously published methods for estimation of the same parameters in human plasma (RINVIK, DYRUD & BRISEID 1966, BRISEID, DYRUD & ARNTZEN 1968).

Key ords Kinins kallikrein

Determinations of parameters of the kinin system in rat plasma have been complicated by the presence of high kininase activity Inactivation of the enzyme by heating at low pH will in addition influence other factors, and the efficiency of the usual kininase inhibitors is disputed In the present work a procedure is described for effective inhibition of the rat plasma kininase by EDTA 2Na. The stabilized plasma preparation thus obtained was used for assaying kininogen fractions and plasma prekallikrein. Rat plasma kininase determinations were also carried out. The proposed methods correspond fairly closely to previously published methods for the estimation of parameters

of the kinin system in human plasma (RINVIK, DYRUD & BRISEID 1966 BRISEID, DYRUD & RINVIK 1967 BRISEID, ARNTZEN & DYRUD 1968 BRISEID, DYRUD & ARNTZEN 1968)

Technique

A. Materials and assays.

Source of rats. Male Wistar albino rats (body weight 180–450 g) obtained from the National Institute of Public Health, Oslo, were used for the preparation of 7 batches of plasma (corresponding to batches 1–7 tables 2 and 3), and male rats (unknown strain, body weight 350–450 g) btained from the Department of Pharmacology and Toxicology The Veterinary College f Norway Oslo, were used for the preparation of 2 batches of plasma (corresponding to batches 8–9 tables 2 and 3).

Rat plasma. From male rats anaesthetized with ether blood was collected from the inferior vena cava into siliconized syringes containing 1 ml of 3.1 % sodium citrate dihydrate solution per 9 ml of blood. The pooled blood from 6 to 20 rats was used for the preparation of batches of rat plasma. The citrated blood was transferred to siliconized centrifuge tubes, and centrifuged at 1.3×10^3 g for 30 minutes at 10 The citrated plasma was stored at −20 as 1 ml samples or used at once for the preparation of kininase, kininogen, or kallikrein preparations.

Rat plasma kininase Citrated plasma was shaken with silica powder (50 mg/ml) for 30 minutes at room temperature (20–24°). The silica was removed by centrifugation and decantation, and the enzyme preparation was stored t −20 as 1 ml samples.

Rat plasma kininogen. 1.00 ml citrated rat plasma was incubated for about 24 hours at 37° with 4.0 mg EDTA 2Na dissolved in 0.04 ml water The kininogen preparation was stored at −20

Rat plasma kallikrein. For activation of prekallikrein 0.25 ml acetone per ml citrated plasma (20 % v/v of acetone) was added, and the mixture left at room temperature (20–24) for about 17 hours. The kininase activity was then eliminated by incubation for about 24 hours at 37 with 4 mg EDTA 2Na per ml of citrated plasma.

Rat urine kallikrein. Urine samples collected from male rats were pooled and centrifuged at 1.3×10^3 g for 10 minutes at 10 The precipitate was removed by decantation and the urine was dialysed against running tap water for about 4 hours. EDTA 2Na (4 mg per ml urine) was subsequently added and the pH adjusted to 7.3 with 1 M sodium hydroxide. After incubation for about 24 hours at 37° the kallikrei preparation was stored at −20

Reagents. Bradykinin Sandoz, A. G Basel, Switzerland. *Ethylenediamine tetra-acetic acid disodium salt* (EDTA-2Na), Thriplex III, Merck, A. G Darmstadt, Germany *Silica powder* "Speed plus Great Lakes, Carbon Corp., Los Angeles, U S. A.

Assays. The kinin determinations were carried out on the isolated rat uterus as "bracketing assays" with a standard dose ratio 3.2. Bradykinin was used as standard.

B Methods.

Determination f rat plasma kininase. The method described by RIEVIK, DYRUD & BRISEID (1966) for human plasma kininase was used.

Determination of the kininogen f action activated by incubation with plasma kallikrein (substrat 1). To 1.00 ml kininogen preparation 1.00 ml of plasma kallikrein preparation was added, and the mixture incubated at 37 for 10 minutes. From the incubate 0.10 ml was withdrawn and transferred to 2.5 ml boiling saline, and the mixture heated for 5 minutes in a boiling waterbath. The mixture was then diluted with saline to 5.00 ml and the kinin solution was assayed at once or kept at 4 for not more than 24 hours before assay

Determination of the kininogen fraction activated by incubation with urine kallikrein. To 1.00 ml kininogen preparation 1.00 ml of a urine kallikrein preparation was added. The further procedure was as described above for determination of substrate 1.

Determination of total kininogen by incubation with (1) acetone and (2) plasma kallikrein. To 1.00 ml kininogen preparation 1.00 ml of a mixture of acetone and saline was added to an acetone concentration of 16.0 % (/v). After incubation for not less than 5 hours at 37 1.00 ml of plasma kallikrein preparation was added and then 0.2 ml saline in order to keep the acetone concentration at 16 % (v/v) and the incubation was then continued for 15 minutes. The reaction was stopped as described above for determination of substrate 1 and the further procedure was also the same.

Determination of total kininogen by incubation with (1) plasma kallikrein and (2) urine kallikrein. The procedure was as described above for the determination of substrate 1 with the following addition. after the 10 minutes incubation with plasma kallikrein 1.00 ml of urine kallikrein preparation was added and the incubation continued for another 10 minutes.

Determination of total kininogen by (1) incubation with acetone and (2) evaporation of the acetone To 1.00 ml kininogen preparation 0.25 ml acetone was added to an acetone concentration of 20 % (v/v). After incubation for 17 hours at room temperature (20–24°) the acetone was evaporated at about 10 mmHg on rotating evaporator and the incubation continued for half an hour The enzyme activity was eliminated as described above for the determination of substrate 1 and the further procedure was also the same.

Determination of rat plasma prekallikrein. To each of 4 one-ml samples of kininogen preparation were added 0.015, 0.025 0.04 and 0.06 ml of kallikrein preparation respectively The kallikrein preparation had previously been diluted with saline 1 + 3 (v/v). To one sample of kininogen preparation 1.00 ml of kallikrein preparation was added. After incubation at 37 for exactly 10 minutes 0.10 ml samples were withdrawn and the reaction stopped as described above for the determination of substrat 1. The further assay procedure was also the same as described above. The kinin values were calculated as μg bradykinin/ml plasma, and the amounts obtained with the 4 submaximal kallikrein concentrations were converted to percentages of the amount obtained with excess of enzyme preparation, i. e. 1 ml/ml substrate. A concentration effect curve was drawn and the amount of kallikrein corresponding to 50 % release was estimated. The kallikrein activities in different enzyme preparations were calculated in terms of standard kallikrein preparation assayed in parallel.

Comments on the technique

A. Materials.

Rat plasma. The citrated rat plasma was prepared from venous blood as previously described for human plasma (BRISEID, DYRUD & ARNTZEN 1968).

Rat plasma kininase was prepared as previously described for human plasma kininase (RINVIK, DYRUD & BRISEID 1966).

Rat plasma kininogen. Opinions differ in the literature as to the efficiency of EDTA 2Na as inhibitor of rat plasma kininase. FERREIRA & ROCHA E SILVA (1962) ROCHA E SILVA, REIS & FERREIRA (1967) and VOGT (1963) found that this substance was not satisfactory while JACOBSEN (1966c) stated that the kininase could be effectively inhibited. According to GAUTVIK & RUGSTAD (1967) the kininase activity in rat plasma was not eliminated by EDTA 2Na, but markedly reduced. An effective inhibition of the kininase in *human* plasma was obtained by means of a 30-minute incubation of the citrated plasma with EDTA 2Na, 4 mg/ml (BRISEID, DYRUD & RINVIK 1967) This contact period was found to be insufficient in *rat* plasma even if the inhibitor concentration was considerably increased. In experiments in which 1.2 µg bradykinin per ml rat plasma kininase preparation was added, the losses of kinin after 24 hours varied from 20 to 30 % for EDTA-2Na-concentrations of 6 to 12 mg per ml. The observed inactivation of

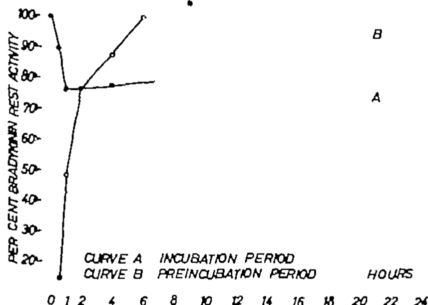


Fig. 1. Inactivation of bradykinin by rat plasma kininase.

Curve A: *Significance of the incubation period* for loss of kinin after preincubation with 6 mg EDTA 2Na per ml citrated plasma for 30 minutes at 37 Incubation of 1.2 µg bradykinin per ml citrated EDTA-plasma at 37 for different periods of time.

Curve B *Significance of the preincubation period with EDTA 2Na*, 4 mg per ml citrated plasma for different periods of time. Incubation of 1.2 µg bradykinin per ml citrated EDTA-plasma at 37° for 24 hours.

kinin occurred early in the incubation period, the kininase being rather quickly inhibited by further contact with the EDTA present. Fig. 1, curve A, shows that the loss of kinin was about the same after 1 hour and after 4 hours at the EDTA 2Na-concentration used, i.e. 6 mg per ml citrated plasma. Fig. 1 curve B, shows that an EDTA-2N -concentration of 4 mg per ml rat plasma kininase preparation was sufficient if the preincubation period was prolonged to 6 hours or more. Experiments with other batches of kininase preparations gave the same results. For practical reasons a preincubation period of 24 hours was chosen for the preparation of rat plasma kininogen. The possibility cannot be excluded however that some alteration in the kininogen might take place during the prolonged preincubation period.

Rat plasma kallikrein was prepared by acetone activation of citrated rat plasma as previously described for human plasma kallikrein (BRISEID, DYRUD & ARNTZEN 1960), but the acetone concentration was increased from 16.7 to 20 % (v/v) to increase the

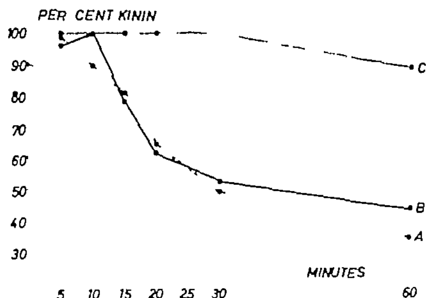


Fig. 2. Inactivation of kinin by kininase in rat urine kallikrein.

Curve A ● ● Incubation of 1.2 µg bradykinin in 1 ml 0.1 M tris buffer of pH 7.3 with 1 ml urine kallikrein preparation.

Curve B ● ——— ● Incubation of 1 ml rat plasma kininogen with 1 ml urine kallikrein preparation. The highest kinin release value observed (at 10 minutes) was adopted as 100 % value.

Curve C ● ● Incubation of 1 ml rat plasma kininogen with 1 ml rat plasma kallikrein preparation for 10 minutes (time: 0 minutes). 1 ml urine kallikrein preparation was then added for full activation of the kininogen and the mixture was further incubated.

For curves B and C the kinin values determined were calculated as µg bradykinin/ml plasma and converted to percentages.

All incubation were carried out t 37

For further details see text.

yield of kininogenase activity and the preincubation period with EDTA 2Na was increased from half an hour to 24 hours in order to inhibit the kininase. To assess the optimum amount of acetone, experiments were carried out with concentrations ranging from 13 to 29 % (v/v). 20 % acetone was found to be most effective, but 18.3 % and 21.6 % acetone yielded only slightly less active kallikrein preparations. With 23 % acetone the activity was about 40 % and with 16.7 % acetone about 20 % of that obtained with the standard acetone concentration, i. e. 20 % (v/v). When the activation was carried out with 13 % or with 29 % acetone, enzyme preparations of very low activities were obtained. As was observed for the activation of human plasma prekallikrein (BAISON, DYRUD & ARNTZEN 1968), the activation period was not very critical. Activation periods with 20 % (v/v) acetone ranging from 10 to 25 hours resulted in almost equiactive kallikrein preparations.

Rat urine kallikrein. The kininase activities of different batches of rat urine kallikrein varied considerably and it was not always possible to block the enzyme completely by means of the suggested treatment with EDTA 2Na. Fig. 4, curve A, shows the loss of kinin in an experiment in which 1.2 μg bradykinin in 1 ml 0.1 M tris buffer of pH 7.3 was incubated with 1 ml of urine kallikrein preparation with some kininase activity. The

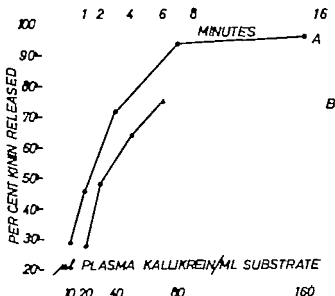


Fig. 3. Rat plasma kallikrein concentration-effect curve and time-effect curve. Kininogen preparation. Rat citrated plasma stabilized with EDTA 2Na, 4 mg/ml plasma. Kallikrein preparation: Rat citrated plasma activated with acetone, 20 % (v/v) for 17 hours at room temperature before addition of EDTA-2Na.
Curve A. Incubation time: 10 minutes
Curve B. Amount of kallikrein preparation/ml kininogen preparation: 60 μl. Kinin released calculated as μg bradykinin/ml plasma and converted to percentages of the amount obtained with an excess amount of kallikrein preparation, 1 ml/ml plasma. For further details see text.

kinin released by urine kallikrein in rat plasma kininogen was also inactivated at the same rate by the urine kininase (fig. 2, curve B). Pretreatment of the kininogen preparation with rat plasma kallikrein, however clearly protected the released kinin against inactivation. Curve C in fig. 2 shows that a fall in kinin activity first took place about 30 minutes after the addition of urine kallikrein when the kininogen had been pretreated for 10 minutes with an excess amount of plasma kallikrein.

B Methods.

Determination of the kininogen fraction activated by incubation with plasma kallikrein (substrate I). When excess amounts of rat plasma kallikrein preparation were added to batches of rat plasma kininogen, amounts of kinin corresponding to about 74 % of the total kininogen were released (table 2, column I). In human plasma the corresponding figure was about 30 % (BRISEID, DYRUD & ARNTZEN 1968). Repeated incubation with fresh enzyme preparation did not increase the yield of kinin. In accordance with the terms used for human plasma the reacting kininogen fraction was called substrate 1 (BRISEID, DYRUD & ARNTZEN 1968 see also JACOBSEN 1966a & b).

In fig. 3 a typical plasma kallikrein concentration-effect curve is shown (curve A). About 100 µl of enzyme preparation per ml substrate caused a release very close to the maximum value obtained with an excess amount of enzyme preparation, i.e. one ml per ml substrate.

Fig. 3 also demonstrates time-effect relationships. Curve B shows that a maximum release effect was obtained after incubation for 6 minutes.

Determination of the kininogen fraction activated by incubation with urine kallikrein. Fig. 2, curve B, shows that a maximum release of kinin caused by an excess amount of urine kallikrein preparation was obtained after an incubation period of 10 minutes. The curve also demonstrates that the kallikrein preparation used contained kininase, which implies that the 100 % value" obtained was correspondingly underestimated. In other urine kallikrein preparations an elimination of the kininase activity was obtained by the preincubation with EDTA.

Determination of total kininogen. The use of the term "total kininogen in the present paper seemed justifiable since methods of determination which were in principle different gave almost identical results (table 2, IIIa and c on the one hand, IIIb on the other), and because similar methods used on human plasma caused full kinin release (BRISEID, DYRUD & RIMVIK 1967 BRISEID, ARNTZEN & DYRUD 1968; BRISEID, DYRUD & ARNTZEN 1968).

Incubation of human citrated EDTA-plasma for 24 hours with acetone in the concentration range of 8-17 % (v/v) caused activation of all the kininogen present (BRISEID, DYRUD & RIMVIK 1967 BRISEID, ARNTZEN & DYRUD 1968 BRISEID, DYRUD & ARNTZEN 1968). Table 1 shows the results of 24-hour experiments carried out with different acetone concentrations in rat plasma. Full kinin release was not obtained for any of the concentrations used. The maximum amounts of released kinin were observed for the acetone concentration range 12 18 % (v/v), and amounted to about 70 % of the total kininogen present. The fact that a maximum kinin release was not obtained in rat plasma by acetone incubation could not be due to the lack of adequate enzyme. If rat plasma kininogen preparations were left with 20 % (v/v) acetone for 17 hours at room temperature (20-24°) and the acetone then evaporated, all the kininogen was activated after incubation for half an hour at 37° (table 2, column IIIc). A considerably shorter acetone contact time sufficed for full kinin release, but the overnight

Table 1

Significance of acetone concentration for the release of kinin in rat plasma.

Rat plasma kininogen (EDTA 2Na, 4 mg/ml citrated plasma and about 24 hours at 37°) was diluted with mixtures of acetone and saline to a final dilution of ⅓ (/v) and then incubated at 37° Aliquots were diluted with boiling saline, heated for 5 minutes at 100 and assayed on the isolated rat uterus. Kinin released was calculated as μg bradykinin/ml plasma.
Kininogen batch number 8, table 2, was used for the experiments. For further details see text.

Acetone conc. % (v/v)	0		8		12		16		18		20		24	
Kinin released	μg	%	μg	%	μg	%	μg	%	μg	%	μg	%	μg	%
1 hours incub.	0.2	6	0.4	13	0.6	19	0.2	6	0.1	3	0.2	6	<0.1	<3
2 hours incub.	0.2	6	1.6	52	2.1	68	1 7	55	0.2	6	0.2	6	<0.1	<3
4 hours incub.	0.3	10	1.8	58	2.1	68	2.3	74	2.1	68	0.3	10	<0.1	<3
6 hours incub.	0.4	13	1.8	58	2.1	68	2.3	74	2.1	68	0.4	13	0.1	3
24 hours incub.	0.9	29	1.8	58	2.1	68	2.3	74	2.1	68	0.7	23	0.1	3

period of 17 hours was chosen for practical reasons. It is probable that the uncompleted release observed when acetone was continuously present was due to the combined effect of two factors: (1). The reversible inhibition of kallikrein by acetone and correspondingly delayed activation of kininogen. Such an effect, which was also observed for human plasma (BRISEID, ARNTZEN & DYRUD 1968), explains the low kinin release value at 20 % acetone (table 1). (2). The presence of higher levels of kininogenase inhibitors, or of inhibitors more resistant to acetone in rat plasma than in human plasma. Thus, for the acetone concentration range 8-18 % (tabl 1) activated kininogenases must have been inhibited before maximum kinin release could be obtained.

When about 70 % of the kininogen had been activated during 5-hour preincubation period with 16 % (v/v) acetone (see table 1), full kinin release could subsequently be obtained by addition of an excess amount of plasma kallikrein (table 2, column IIIa).

Determination f rat plasma prekallikrein. The procedure was similar to that previously described for human plasma (BRISEID, DYRUD & ARNTZEN 1968) with the modification that acetone was not added. This means that acetone-susceptible inhibitors might influence the results if present in different amounts in the "test- and the "standard" preparations.

Results

Rat plasma kininogen.

Table 2 shows the results of determinations of kininogen activated by rat plasma kallikrein and by rat urine kallikrein and also the results of determinations of total kininogen obtained by different methods.

Table 2

Determination of kininogen in rat plasma.

I. The kininogen fraction activated by an excess amount of rat plasma kallikrein preparation.

II. The kininogen fraction activated by an excess amount of rat urine kallikrein preparation. The enzyme preparation used did not contain any significant amounts of kininase.

III. Total kininogen activated by
  a) acetone (16 % v/v) and then rat plasma kallikrein.
  b) rat plasma kallikrein and then rat urine kallikrein.
  c) acetone (20 % v/v) and then evaporation of the acetone.

The first 7 and the last 2 batches of kininogen were prepared from plasma specimens obtained from rats of different origin.
For details see text.

Kininogen batch	Rats		Kinin released/ml plasma as bradykinin						
	Number	Weight range g	I		II		IIIa	IIIb	IIIc
			μg	%	μg	%	μg	μg	μg
1	20	350–380	1.4	73	1.5	78	1.8	1.9	2.1
2	8	210–260	1.5	80	1.4	75	1.9	1.9	1.8
3	6	180–200	1.4	69	–		1.9	2.0	2.2
4	8	180–210	1.4	73	1.4	73	1.9	2.0	1.9
5	20	350–450	1.5	70	1.4	66	2.2	2.1	2.1
6	8	180–225	1.4	70	1.3	65	2.0	1.9	2.1
7	20	310–370	1.7	82	1.4	68	2.0	2.1	2.1
Mean			1.47	73.9	1.40	70.8	1.96	1.99	2.04
8	15	350–450	2.1	69	–	–	3.1	3.0	–
9	15	about 350	2.0	80	1.5	60	2.4	2.6	2.5
Mean			2.05	74.5	–	–	2.75	2.80	–

The different methods used for determination of total kininogen in rat plasma gave very similar figures, 2.0 μg/ml for the first 7 batches and 2.8 μg/ml for the last 2 batches. The kininogen activated by plasma kallikrein and by urine kallikrein corresponded to average releases in the first 7 kininogen batches of 1.5 and 1.4 μg/ml plasma respectively (74 and 71 %), calculated as bradykinin. The average release value obtained by plasma kallikrein in two kininogen batches of different origin (8 and 9) were significantly higher i.e. 2.1 μg/ml, but of the same order of magnitude when compared with the value for total kininogen, i.e. 75 %. All percentages given in table 2 are calculated from the average values for total kininogen obtained by methods IIIa, b, and c.

Table 3

Determination of kininase and prekallikrein in rat plasma.

I. Plasma kininase preparation. Citrated plasma treated with silica powder for 30 minutes. Test solution. Kininase preparation + 1.2 µg bradykinin dissolved in 0.12 ml saline + 0.1 M tris buffer of pH 7.3 to 1 ml. Remaining bradykinin after 8 minutes at 37° was determined.

II. The individual batches of kallikrein preparations were tested for kinin releasing activity in comparison with standard batch.

The kininase and the prekallikrein batch numbers refer to the same plasma batches as the kininogen batch numbers in table 2.

For further details see text.

Kininase and prekallikrein batch	I µl plasma kininase preparation causing 50 % inactivation	II µl kallikrein preparation per ml plasma substrate causing 50 % kinin release		
		Test	Standard	Relative/activity Test/Standard
1	27	–	–	–
2	27	38	40	1.1
3	28	18	21	1.2
4	25	17	17	1.0
5	32	23	24	1.0
6	26	25	25	1.0
7	24	–	–	–
Mean	27.0	24.2	25.4	1.06
8	–	44	36	0.8
9	37	38	36	0.9
Mean	–	41.0	36.0	0.85

The experiments shown in table 4 were carried out in order to provide evidence of the existence in the rat plasma kallikrein preparation of two different kallikrein fractions. The results will be dealt with under "Discussion

Rat plasma kininase.

Table 3 gives the results of determinations of the plasma kininase activities in 8 batches of kininase preparations. Taking into account the 7 batches from rats of the same origin, an average amount of 27 µl kininase preparation was required to inactivate a standard amount of bradykinin under standard conditions. Previously it was found that 170 µl of human plasma kininase preparation had the same effect (RINVIK, DYRUD & BRISEID 1966), which means that rat plasma is about 6 times more active.

Rat plasma prekallikrein

Table 3 shows the results of determinations of 7 batches of acetone activated rat plasma kallikrein It is obvious that the considerable day-to-day variations made it necessary to use a standard enzyme preparation tested in parallel.

Discussion

Rat plasma kininogen.

Previous authors have reported significantly higher figures for total kininogen in rat plasma than those obtained in the present work, 2 µg/ml for the first 7 batches and 2.8 µg/ml for the last 2 batches. Thus DINIZ & CARVALHO (1963) found 4 µg/ml plasma GREEFF SCHARNAGEL, LÜHR & STROBACH (1966) about 12 µg/ml DAWSON STARR & WEST (1966) about 5 µg/ml and ROCHA E SILVA, REIS & FERREIRA (1967) about 5 µg/ml. In all the papers referred to, however the method of DINIZ & CARVALHO (1963) was used (or that method with minor modifications) in which the plasma substrate was prepared by heating at 100° and at a low pH and the kinin then released by incubation with trypsin. ROCHA E SILVA, REIS & FERREIRA (1967) also examined the release of kinin in plasma specimens which were not heated or acid-treated and found a release of about 4 µg/ml for trypsin, about I µg/ml for activation with glass powder and a still lower release value when 10 / acetone was used.

The releases of kinin observed by previous authors for plasma kallikrein and for glandular kallikrein were considerably lower than the values reported in the present paper Thus JACOBSEN (1966c) found kinin activity corresponding to about 0 4 µg bradykinin/ml plasma when a human or a rat plasma kallikrein preparation was used, and 0 2 µg bradykinin/ml plasma when rat saliva or rat urine was used. Later GAUTVIK & RUGSTAD (1967) found kinin corresponding to about 0 2 µg bradykinin/ml plasma when they used a human pseudoglobulin preparation and roughly the same amount of kinin when rat saliva was used as enzyme preparation. It should be mentioned that JACOBSEN (1966c) added the kininase inhibitor EDTA 2Na just before the addition of kinin-releasing enzyme while GAUTVIK & RUGSTAD (1967) used a 5-minute preincubation period of inhibitor and plasma substrate.

The fact that the amounts of kinin released by plasma kallikrein when added to the amounts released by urine kallikrein far exceeded the total kininogen present might indicate the presence of 3 different kininogen fractions in rat plasma. The results of further experiments on this question are to be published separately (BRISEID, DYRUD & ARNTZEN 1970)

Two kallikrein fractions.

The term plasma kallikrein has been used in the present paper to indicate all plasma kininogenases activated by acetone. The inactive enzyme precursors were called prekallikrein. No distinction could be made between enzymes acting indirectly and enzymes acting directly on the kininogen by using substrate heated at 60° for one hour to denaturate the prekallikrein. Unpublished experiments showed that such a heating procedure also altered the kininogen to the extent that it reacted neither with rat plasma kallikrein nor with human plasma kallikrein.

Tables 2 and 4 provide evidence of the presence of two different kallikrein fractions in acetone activated rat plasma. When a plasma kininogen preparation was first incubated with a sufficiently high concentration of acetone for a sufficiently long period of time (in table 2, column IIIa, 16 / v/v for 5 hours) the subsequent addition of an excess amount of plasma kallikrein rapidly caused full kinin release. Maximum release was also obtained from

Table 4

Significance of the elimination by acetone of kininogenase inhibitors for a maximum release f kinin in rat plasma

RP-kall. = Rat plasma kallikrein preparation.
RP kin. = Rat plasma kininogen preparation.
Rat plasma kallikrein was added in an excess amount to the kininogen preparation, 1 ml per ml (Exp. I and II). When kininogen preparation was added in the second part of the experiments, a volume of 1 ml per ml of the kininogen preparation already present was used (Exp. I, II and III).
While the acetone treatment took place at room temperature (20-24°), all other incubations were carried out at 37°
Batch number 7 in table 2 was used for the experiments.
For further details see text.

Experiment	Treatment of *rat plasma kininogen* preparation with	Kinin released per ml plasma as µg bradykinin after incubation time in hours: ½ µg	½ %	1 µg	1 %	2½ µg	2½ %
I	1. 20 % /v acetone for 17 hours and evapor of acetone	2.1	100				
	2. RP-kin.			1.7	81	1.7	81
II	1. RP kall.	1 7	81				
	2. RP kin.			1.7	81		
III	1 RP kall.	1.7	81				
	2. RP-kin.					1.4	67

acetone activation of the prekallikrein in the plasma kininogen preparation with 20 % (v/v) acetone and subsequent evaporation of the acetone (table 2, column IIIc). However, when fresh substrate was then added, only the presence of kininogenases capable of activating the main kininogen fraction (substrate 1) could be demonstrated. Experiment I in table 4 shows that an amount of kinin corresponding only to that fraction was released.

The above results suggest that a kallikrein fraction responsible for the activation of the minor kininogen was rapidly inactivated, in the absence of acetone, by inhibitors present in the rat plasma kininogen preparation. These inhibitors were irreversibly eliminated during a 17 hour contact period with 20 % (v/v) acetone, while a 5 hour contact period with 16 % acetone also reduced the inhibitor level considerably. Experiments II and III in table 4 make it unlikely that one single kallikrein fraction reacts only slowly with the minor kininogen. The enzyme in plasma kallikrein acting on the main kininogen was still capable of causing full kinin release from the major kininogen fraction in freshly added substrate after contact for half an hour with the inhibitors of the rat plasma kininogen preparation. After a 2-hour contact period the remaining enzyme activity still released kinin corresponding to about 80 % of the main kininogen fraction.

REFERENCES

Briseid, K., O. K. Dyrud & S. F. Rinvik. *In vitro* estimation of the rate of release of kinin in human plasma. *Acta pharmacol. et toxicol.* 1967, 25, 201–213.

Briseid, K., O. K. Dyrud & F. C. Arntzen. Determination of prekallikrein in human plasma. *Acta pharmacol. et toxicol.* 1968, 26, 395–401.

Briseid, K., F. C. Arntzen & O. K. Dyrud. Studies on the release of kinin by acetone in human plasma. *Acta pharmacol. et toxicol.* 1968, 26, 402–412.

Briseid, K., O. K. Dyrud & F. C. Arntzen. Substrates for kinin-releasing enzymes in rat plasma. *Acta pharmacol. et toxicol.* 1970, 28, 138–144.

Dawson, W., M. S. Starr & G. B. West. Inhibition of anaphylactic shock in the rat by antihistamines and ascorbic acid. *Brit. J. Pharmacol.* 1966, 27, 249–255.

Diniz, C. R. & I. F. Carvalho: A micromethod for determination of bradykininogen under several conditions. *Ann. N. Y. Acad. Sci.* 1963, 104, 77–89.

Ferreira, S. H. & M. Rocha E Silva. Potentiation of bradykinin by dimercaptopropanol (BAL) and other inhibitors of its destroying enzyme in plasma. *Biochem. Pharmacol.* 1962, 11, 1123–1128.

Gautvik, K. M. & H. E. Rugstad. Kinin formation and kininogen depletion in rats after intravenous injection of ellagic acid. *Brit. J. Pharmacol.* 1967, 31, 390–400.

Groeff, K., K. Scharnagel, R. Lühr & H. Strobach: Die Abnahme des Kininogengehaltes des Plasmas beim toxischen, anaphylaktischen und anaphylaktoiden Schock. *Arch. exp. Path. Pharmakol.* 1966, 253, 235–245.

Jacobsen, S. Separation of two different substrates for plasma kinin-forming enzymes. *Nature* 1966a, 210, 98–99.

Jacobsen, S. Substrates for plasma kinin-forming enzymes in human, dog, and rabbit plasmas. *Brit J Pharmacol.* 1966b, 26 403–411

Jacobsen, S. Substrates for plasma kinin-forming enzymes in rat and guinea-pig plasmas. *Brit J Pharmacol.* 1966c, 28, 64–72.

Rinvik, S. F O K. Dyrud & K. Briseid. Determination of plasma kininogen, plasma kininase and erythrocyte kininase. *Acta pharmacol. et toxicol.* 1966, 24, 169–178.

Rocha E Silva, M., M. L. & S. H. Ferreira. Release of kinins from fresh plasma under varying experimental conditions. *Biochem. Pharmacol.* 1967 16, 1665–1676.

Vogt, W Spontane Plasmakininbildung und Hemmung des Kininabbaus in Calciumfreiem Plasma. *Arch. exp Path. Pharmakol.* 1963, 245, 286–287

Acta pharmacol. et toxicol. 1970 28 138–144

From the Department of Pharmacology Institute of Pharmacy
University of Oslo, Norway

Substrates for Kinin-Releasing Enzymes in Rat Plasma

By

Kjell Briseid, Olav Kjell Dyrud and Finn Cato Arntzen

(Received September 5 1969)

Abstract Indirect evidence has been provided for the presence of 3 kininogen fractions. The average amounts of kinin released by rat plasma kallikrein (1.5 µg/ml plasma, S. E. M. = 0.03) and by rat urine kallikrein (1.4 µg/ml plasma, S. E. M. = 0.03) in 7 plasma batches corresponding to a total of 90 rats, when added up significantly exceeded the total kininogen (2.0 µg/ml plasma, S. E. M = 0.04) Methods and materials were as described by Briseid, Dyrud & Öie (1970). It is suggested that plasma kallikrein released kinin from 2 kininogen fractions, S1' and S1 and that urine kallikrein released kinin from 2 kininogen fractions, S1 and S2. Repeated incubation with each of the kininogenase preparations used did not increase the yield of kinin. Soybean trypsin inhibitor did not reduce the amount of kinin released by urine kallikrein the plasma kallikrein, however was strongly inhibited. In control experiments leucine aminopeptidase transformed kallidin to bradykinin, but did not increase the kinin activity of the urine kallikrein incubates.

Key words. Kinins – kallikrein.

Two substrates for plasma kinin-forming enzymes were detected in rat plasma by Jacobsen (1966c) One of the kininogen fractions (substrate 1) yielded kinin on treatment with plasma kallikrein (a pseudoglobulin preparation) as well as with glandular kallikreins, while only glandular kallikreins were found to be active on the other kininogen fraction (substrate 2) In connection with our work on methods for determining the factors involved in the kinin system in rat plasma (Briseid, Dyrud & Öie 1970), the release of kinin by rat plasma kallikrein (acetone-activated) and by glandular kallikrein (rat urine kallikrein) was examined. The fact that the amounts of kinin released by one of the kininogenase preparations, when added to the amounts released by the other significantly exceeded the total kininogen present, might indicate the presence in rat plasma of 3 different kininogen fractions, one of which reacted with both types of enzyme preparation. In the present work such a possibility was further examined.

Technique

Most of the materials and methods used were as described previously (BRISEID, DYRUD & ÖIE 1970). In addition to the chemicals mentioned there, the following substances were used in the present work: *Leucine aminopeptidase* and *Soybean trypsin inhibitor* both preparations were obtained from Worthington Biochemical Corp Freehold, New Jersey U S. A.

A pseudoglobulin preparation was made from human plasma by a procedure described by LEWIS (1958) and later used by JACOBSEN (1966a & b) and GAUTVIK & RUGSTAD (1967).

Results

Table 1 shows the results of experiments carried out to provide, if possible, indirect evidence for the presence of 3 different kininogen fractions in rat plasma. Two batches of rat plasma kininogen preparation were tested in each experiment.

In experiments I and II of table 1 the kinin releases obtained with rat plasma kallikrein and with rat urine kallikrein were determined, as well as the release values obtained by the combined use of the two kininogenase preparations, and corresponding to the total kininogen (BRISEID, DYRUD & ÖIE 1970)

Table 1

Determination of the amounts of kinin released in rat plasma by incubation with different kininogenase preparations.

Batches of rat plasma kininogen preparation.

A. Prepared from 20 rats, weight range 310–370 g.

B. Prepared from 11 rats, weight range 255–275 g.

C. Prepared from 8 rats, weight range 180–225 g.

Kininogenase preparations:

RP-kall. Acetone activated rat plasma kallikrein.

RU-kall. Rat urine kallikrein.

PS-glob. Human pseudoglobulin.

The kininogenase preparations were used in excess amounts, 1 ml per ml kininogen preparation for RP-kall. and RU-kall., 2 ml per ml kininogen preparation for PS-glob.

Saline. 0.9 % sodium chloride solution was added to dilution of the kininogen preparation of 1 + 4 (v/v).

LAP Leucine aminopeptidase was used in an amount which rapidly converted 2 µg kallidin in 2 ml 0.1 M tris buffer (containing 4 mg EDTA – 2 N per ml) into bradykinin.

SBTI Soy bean trypsin inhibitor 0.5 mg per ml kininogen preparation was used.

All incubations were carried out at 37°

Released kinin was tested on the isolated rat uterus.

For further details see text, and BRISEID, DYRUD & ÖIE (1970).

Experiment	Rat plasma kininogen preparation: Batch	Rat plasma kininogen preparation: Treatment	Kinin released/ml plasma as bradykinin after incub. time in minutes: 10 µg	10 %	20 µg	20 %	40 µg	40 %
I	A	1 RP kall.	1.9	79				
		2. RU-kall.			2.4	100		
	B		1.3	62	2.1	100		
II	A	1. RU-kall.	1.4	58				
		2. RP kall.			2.4	100		
	B		1.2	57	2.1	100		
III	A	1 RP kall.	1.9	79				
		2. RP-kall			2.0	83		
	B		1.6	76	1.5	71		
IV	A	1 RU-kall.	1.4	58				
		2. RU-kall.			1.6	67		
	B		1.1	52	1.2	57		
V	B	1. RU-kall.	1.1	52				
		2. LAP					1.1	52
	C		1.5	75			1.6	80
VI	B	1. RP kall.	1.6	76				
		2. LAP					1.7	81
	C		1.5	75			1.4	70
VII	A	SBTI + RP-kall.	0.3	13			0.3	13
	B		0.3	14			0.3	14
VIII	C	SBTI + RU-kall.	1.4	70				
	B		1.2	57				
IX	A	Saline			0.5	21	0.9*	38
X	A	SBTI + Saline			0.2*	8	0.2*	8
XI	A	1 PS-glob.	1.9	83				
		2. RU-kall.			2.3	100		
	B		1.4	70	2.0	100		
XII	A	1 RU-kall.	1.4	61				
		2. PS-glob.			2.1	91		
	B		1.2	60	1.8	90		

* 15 minutes incubation.
** 60 minutes incubation

In experiment III an additional incubation of kininogen preparation with plasma kallikrein did not increase the yield of kinin. This result shows that

the observed cessation of kinin release at a level of 70 to 80 / of the total kininogen was not due to the effect of kininogenase inhibitors present, but must be ascribed to the presence of at least 2 different kininogen fractions. The results with rat urine kallikrein in experiment IV also demonstrate the lack of homogeneity of the rat plasma kininogen.

According to WEBSTER & PIERCE (1963) human urinary kallikrein will set free the decapeptide kallidin in human plasma, while plasma kallikrein (acetone activated) releases bradykinin only Aminopeptidases present in human plasma, and also in human urine, will, however rapidly convert kallidin to bradykinin. As the rat uterus method was used in the present work for the assays of released amounts of kinin, the considerable difference in uterine activity possessed by the two polypeptides mentioned above made experiments V and VI of table 1 necessary The results show that LAP (leucine aminopeptidase), which has previously been found to transform kallidin to bradykinin (BRISEID, DYRUD & RINVIK 1966) did not increase the activity of the kinin released in rat plasma by a rat urine kallikrein preparation or by a rat plasma kallikrein preparation.

It is generally accepted that SBTI (soy bean trypsin inhibitor) will inhibit plasma kallikrein, but not glandular kallikreins. The results of experiments VII and VIII in table 1 when compared with the results of experiments I and II, show in agreement with this, that SBTI inhibited the rat plasma kallikrein preparation used, but not the rat urine kallikrein preparation.

Experiments IX and X of table 1 show that a considerable kinin release took place on dilution of the rat plasma kininogen preparation with saline (1 + 4) and subsequent incubation at 37 for 20 minutes. In the presence of SBTI the extent of release was markedly reduced.

In experiments XI and XII of table 1 the kinin releases obtained with a human plasma pseudoglobulin preparation and with rat urine kallikrein were determined, as well as the release values obtained by the combined use of the two enzyme preparations. When comparing the results of experiments XI and XII with the results of experiments I and II, it is evident that no distinction could be made between the effect of the human pseudoglobulin preparation and the rat plasma kallikrein preparation.

Table 2 shows the results of determinations of the amounts of kinin released by rat plasma kallikrein, by rat urine kallikrein, and by the combined use of the two enzyme preparations. The data shown in columns I, II, and III have been previously published (BRISEID, DYRUD & ØYE 1970) and the kininogen batch numbers in table 2 correspond to the numbers given in that paper In table 2, the total kininogen has been divided into 3 different fractions on the assumption that rat plasma kallikrein releases kinin from 2 kininogen fractions, S1 and S1 and rat urine kallikrein from 2 fractions, S2 and S1 It can be seen that the kininogen fraction specific for plasma

Table 2

Determination of kininogen fractions in rat plasma.

I The kininogen fraction activated by an excess amount of rat plasma kallikrein preparation.
II The kininogen fraction activated by an excess amount of rat urine kallikrein preparation.
III. Total kininogen activated by rat plasma kallikrein and rat urine kallikrein.

The hypothetical kininogen fractions S1 and S2 were calculated as the differences between the data in columns III and II and in III and I respectively. The remaining kininogen fraction was thus S1.

Kininogen batch	Kinin released/ml plasma, as bradykinin								
	I	II	III	S1		S1		S2	
	µg	µg	µg	µg	%	µg	%	µg	%
1	1.4	1.5	1.9	0.4	21	1.0	53	0.5	26
2	1.3	1.4	1.9	0.5	26	1.0	53	0.4	21
3	1.4	–	2.0	–	–	–	–	0.6	30
4	1.4	1.4	2.0	0.6	30	0.8	40	0.6	30
5	1.5	1.4	2.1	0.7	33	0.8	40	0.6	29
6	1.4	1.3	1.9	0.6	32	0.8	42	0.5	26
7	1.7	1.4	2.1	0.7	33	1.0	48	0.4	19
Mean	1.47	1.40	1.99	0.58	29	0.90	46	0.51	26

kallikrein, S1 contributed 29 % to the total kininogen the fraction specific for the glandular kallikrein, S2, was 26 % while the common substrate, S1 amounted to 46 % of the total kininogen.

Discussion

The combined amounts of kinin released in rat plasma by rat plasma kallikrein and by rat urine kallikrein, significantly exceeded the kinin released by the combined use of the enzyme preparations (table 1 experiments I and II). This means that the two crude kininogenase preparations used, whether they acted directly on the kininogen or by an indirect mechanism, must have partly activated different kininogen fractions, but also partly activated the same fraction. The hypothesis can be put forward that 3 different kininogen fractions were present.

With regard to this assumption, it can be objected that one or both of the kininogenase preparations used might have contained enzymes capable of exhausting all the kininogen present, the release process, however being brought to an end by inhibitors present in the substrate. If this were so, the

presence of two kininogen fractions only would suffice to explain the results mentioned above The fact, however that repeated incubatron of the substrate with each of the kininogenase preparations used did not increase the yield of kinin (table 1 experiments III and IV) demonstrated that the kininogenase inhibitors present were not responsible for the observed cessation in kinin release. This conclusion does not contradict the previously published suggestion that inhibitors present in the substrate will inactivate one kininogenase fraction in the acetone-activated plasma kallikrein so rapidly that one kininogen fraction present in the substrate will not be activated at all (BRISEID, DYRUD & ÖIE 1970)

The possibility that the crude urine kallikrein preparation used was either contaminated with plasma kallikrein, or acted through activation of plasma kallikrein, was excluded by experiments in which it was shown that SBTI did not reduce the amount of kinin released by the glandular kallikrein preparation. The plasma kallikrein preparation, on the other hand, was almost completely inhibited (table 1 experiments VII and VIII)

Under the conditions used in the present work (negligible dilution, siliconized glass, and relatively short incubation periods) the "spontaneous activation of kininogenases was insignificant. In addition, the fact that SBTI markedly inhibited the kinin release observed on incubation of rat plasma kininogen preparation diluted with saline 1 + 4 (v/v) (experiments IX and X in table 1) demonstrated that a parallel running dilution effect did not contribute to the extent of release obtained with rat urine kallikrein.

It has previously been demonstrated (BRISEID DYRUD & ÖIE 1970) that the kininogenases activated in rat plasma by acetone were capable of causing release of kinin from all the kininogen present, provided plasma kininogenase inhibitors had previously been destroyed by acetone. In view of the theory of 3 kininogen fractions suggested in the present paper this means that plasma kallikrein can also release kinin from substrate 2, but only if the plasma inhibitors of the actual kininogenase fraction have been eliminated. As the plasma kininogenase fraction still active in the presence of the inhibitors proved to be capable of full kinin release from the other 2 kininogen fractions, we decided to keep the term S1 for both fractions, but marked for the fraction specific for plasma kallikrein and double marked for the fraction which also released kinin on incubation with urine kallikrein.

According to JACOBSEN (1966c) both kininogen fractions identified by him in rat plasma (substrates 1 and 2) released kinin on incubation with rat saliva or rat urine kallikrein. The plasma kallikrein preparation mainly used by him, a pseudoglobulin preparation made from human plasma, released kinin only from his substrate 1

From our results it seemed possible that the proposed kininogen fraction S1 which was activated by both types of kallikreins, might be identical with

Jacobsen s substrate 1 A pseudoglobulin preparation was accordingly prepared by the procedure used by Jacobsen to examine if this enzyme preparation might release kinin from substrate 1 only Experiments XI and XII of table 1 however demonstrate that under the conditions used the preparation behaved like our acetone-activated plasma kallikrein (experiments I and II), releasing kinin from the entire substrate 1

Conclusion The present experiments provide indirect evidence in support of the theory that the rat plasma kallikrein preparation used, released kinin from a kininogen fraction not activated by rat urine kallikrein (substrate 1') as well as from another fraction which was also activated by urine kallikrein (substrate 1) The urine kallikrein preparation on the other hand, released kinin from a kininogen fraction (substrate 2) not activated by the plasma kallikrein preparation (under the conditions used) but also from a kininogen fraction activated by plasma kallikrein (substrate 1 ') The possibility cannot be entirely excluded that the glandular kallikrein acted indirectly on the last-mentioned substrate, through activation of a plasma kininogenase. If this was so the hypothetical kininogenase was not inhibited by SBTI. To our knowledge the presence of plasma kallikreins not inhibited by SBTI has not been demonstrated, and a direct effect by urine kallikrein on the common substrate S1 is the more probable explanation.

REFERENCES

Briseid, K., O K. Dyrud & S. F Rinvik: The release of kinins in human plasma substrates. *Acta pharmacol. et to icol.* 1966, 24 39–49

Briseid, K., O. K. Dyrud & S. Ole: Determination of factors of the kinin system in rat plasma. *Acta pharmacol. i to icol* 1970, 28, 124–137

Gautvik, K. M. & H. E. Rugstad. Kinin formation and kininogen depletion in rats after intravenous injection of ellagic acid. *Brit. J Pharmacol.* 1967 31, 390–400.

Jacobsen, S.. Separation of two different substrates for plasma kinin-forming enzymes. *Nature* 1966a, 210 98–99

Jacobsen, S., Substrates for plasma kinin-forming enzymes in human, dog, and rabbit plasmas. *Brit. J Pharmacol.* 1966b, 26 403–411.

Jacobsen, S.. Substrates for plasma kinin-forming enzymes in rat and guinea-pig plasmas. *Brit J Pharmacol.* 1966c, 28 64–72.

Lewis, G P Formation of plasma kinins by plasmin. *J Physiol.* 1958, 140 285–300.

Webster M. E. & J V Pierce: The nature of the kallidins released from human plasma by kallikreins and other enzymes. *Ann. N Y Acad. Sci.* 1963, 104 91 105.

Acta pharmacol. et toxicol. 1970, **28** 145–148

From the Institute of Experimental Medicine and Surgery
University of Montreal, Quebec, Canada

Prevention of Mephenesin Intoxication by Catatoxic Steroids

By

Hans Selye

(Received November 17 1969)

Abstract The purpose of these experiments was to determine whether pretreatment with certain steroids could protect against mephenesin poisoning. In rats, the muscular paralysis (gaged in terms of an arbitrary scale) and the mortality induced by mephenesin (30–60 mg/100 g body weight given subcutaneously) are inhibited by various catatoxic steroids such as ethyloestrenol, SC 11927 spironolactone and norbolethone (10 mg/100 g body weight twice daily by stomach tube) beginning four days before treatment with the muscle relaxant. Conversely under comparable conditions, steroids previously shown to possess little or no catatoxic potency in other test systems (prednisolone, progesterone, oxandrolone, triamcinolone, desoxycorticosterone, hydroxydione) exhibited no protective effect under the same conditions. Apparently the protective effect of various steroids against mephenesin intoxication parallels their catatoxic potency in other tests.

Key-words: mephenesin – steroids.

Mephenesin is one of the most extensively studied centrally acting muscle relaxants and is considered to be the prototype of the drugs belonging to this class. The liver is the major site of mephenesin degradation and its pharmacologic effects parallel the concentration of the free drug in the plasma (Morrison *et al.* 1948 Wyngaarden *et al.* 1949) In view of these facts, it seemed of interest to establish whether mephenesin intoxication could be combated by catatoxic steroids whose protective effect depends, at least to a large extent, on their ability to accelerate the hepatic detoxication of drugs (Selye 1969)

Materials and Methods

145 female Holtzman rats with mean initial body weight of 100 g (range 90–110 g) which were maintained exclusively on Purina Laboratory Chow and tap water were divided into 11 groups and treated as outlined in tabl 1. In order to obtain the best prophylactic effect, it is important to allow a few days of pretreatment with catatoxic

steroids hence, all animals received mephenesin (Squibb) beginning only on the 4th day after the initiation of the steroid treatment. On that day 30 mg and on the following day 60 mg of mephenesin were administered per 100 g body weight subcutaneously always in 0.2 ml of propylene glycol.

The following steroids were tested. ethyloestrenol (Organon)· SC 11927 [that is, potassium 3-(3-oxo-9α-fluoro-11β, 17β-dihydro-4-androstene 17α-yl (Searle)· spironolactone (Searle) norbolethone (Wyeth) oxandrolone (Searle)· prednisolone acetate (Roussel) progesterone (Schering)· triamcinolone (fluorprednisolonum NFN) (Lederle); desoxycorticosterone acetate (desoxicortonum NFN) (Ciba) and hydroxydione sodium hemisuccinate (Pfizer). These steroids were administered at the dose of 10 mg in 1 ml of water twice daily by stomach tube· only in the case of triamcinolone did we have to reduce the dose to 2 mg, since fatal intercurrent infections tend to develop with larger amounts of this highly potent glucocorticoid. The controls received 1 ml of water twice daily by stomach tube.

The severity of the resulting paralysis was recorded 150 min. after the second mephenesin injection in terms of an arbitrary scale in which 0 = no lesion, 1 = just detectable, 2 = moderate, 3 = most pronounced, as previously described (SELYE *et al.* 1969). However for statistical evaluation, we recognized only two degrees: minor and sometimes dubious degrees of paralysis (between "O" and + in our scale) were rated as negative, all others as positive. These data, as well as the mortality rates, were then arranged in a 2 × 2 contingency table and the statistical significance computed by the "Exact Probability Test of FISHER & YATES (FINNEY 1948 SIEGEL 1956). The experiment was terminated on the 2nd day of mephenesin treatment and the mortality rates in table 1 are based on the number of animals that died by that time.

Results and Discussion

As shown in table 1 with the high dose used here, mephenesin produced extremely severe muscular paralysis in nine out of ten control animals but in none of those pretreated with ethyloestrenol, SC-11927 spironolactone or norbolethone. On the other hand, oxandrolone, prednisolone, progesterone, triamcinolone, desoxycorticosterone acetate and hydroxydione exhibited little, if any protective effect. Since in this acute experiment, the mortality rate was only 30 / in the controls, the protective action of the catatoxic steroids was not significant. Yet, it is perhaps worthy of note that there was no mortality in any of the groups treated with the four compounds that proved to be most potent in preventing paralysis.

Perhaps the most striking result of these investigations was to show that the steroids previously found to protect against many other types of intoxication (SELYE 1969 SELYE *et al* 1960 1965 & 1969) are also most potent in inhibiting mephenesin-induced paralysis. Here, as in our previous work, the catatoxic action was independent of the other pharmacologic properties of the steroids examined Some of the active catatoxic compounds are anabolics (ethyloestrenol, norbolethone) while others are antimineralocorticoids (SC 11927 spironolactone), virtually devoid of other properties. On the other hand, no such catatoxic effect was associated with high mineralocorticoid

Table 1

Effect of catatoxic steroids on mephenesin intoxication.

Group	Treatment[1]	Paralysis[2]	Mortality[2] (%)
1	None	9/10	30
2	Ethyloestrenol	0/10**	0 NS
3	SC 11927	0/15	0 NS
4	Spironolactone	0/10**	0 NS
5	Norbolethone	0/10**	0 NS
6	Oxandrolone	8/15 NS	7 NS
7	Prednisolone acetate	7/15	0 NS
8	Progesterone	8/15 NS	27 NS
9	Triamcinolone 2 mg	15/15 NS	40 NS
10	Desoxycorticosterone acetate	7/15*	13 NS
11	Hydroxydione	8/15 NS	13 NS

[1]) In addition to the agents listed in this column, the rats of all groups were given mephenesin as indicated in the text. The readings were made on the second day 150 min. after mephenesin administration.
[2]) ** = $P < 0.005$ * = $P < 0.05$ NS = not significant.

(desoxycorticosterone), glucocorticoid (triamcinolone) or anaesthetic (hydroxydione) potency

The most active catatoxic steroids, e g. spironolactone (KOVÁCS *et al* 1969) and norbolethone (GARDELL *et al* 1969), stimulate the proliferation of the smooth endoplasmic reticulum in hepatocytes, a feature allegedly characteristic of inducers of microsomal drug-metabolizing enzymes in general (REMMER & MERKER 1963) Furthermore, it has been shown that spironolactone norbolethone and ethyloestrenol, in doses at which they inhibit barbiturate anaesthesia, increase the production of barbiturate-metabolizing enzymes, as shown by incubation of the hepatic microsomal fraction with this substrate (SOLYMOSS *et al* 1969) There is no proof that all catatoxic actions depend on the induction of drug-metabolizing enzymes in the hepatic microsomes but, at least in several cases, this mechanism appears to be chiefly responsible for their protective effect.

Acknowledgements

This work was supported by the Ministère de la Santé, Québec, and by the Medical Research Council of Canada (Block Term Grant MT 1829).

The author wishes to thank the following Companies for kindly supplying the compounds used in this experiment: Ciba Co Ltd., (desoxycorticosterone acetate) G D Searle & Co. (SC 11927 spironolactone and oxandrolone),

E. R. Squibb & Sons Ltd., (mephenesin), Pfizer Ltd (hydroxydione) Lederle Laboratories (triamcinolone) Organon Inc. (ethylestrenol) Schering Corp. (progesterone) and Wyeth Laboratories (norbolethone)

REFERENCES

Finney D. J.: The Fisher Yates test of significance in 2 × 2 contingency tables. *Biometrika* 1948, 35 145–156.

Gardell, C., J A. Blascheck & K. Kovács: Action de la norbolethone sur le réticulum endoplasmique hépatique du rat. *J Micr* 1970, in press.

Kovács, K., J A. Blascheck & C. Gardell Spironolactone-induced proliferation of smooth-surfaced endoplasmic reticulum in the liver of rats. *Z. ges. exp Med.* 1970, in press.

Morrison, J L., P Saperstein, H. A. Walker & A. P Richardson: Distribution and rate of disposition of myanesin in dogs. *Proc. Soc. exp. Biol. Med.* (N Y.) 1948, 68, 339–343.

Remmer H. & H J Merker: Enzyminduktion und Vermehrung von endoplasmatischem Reticulum in der Leberzelle während der Behandlung mit Phenobarbital (Luminal). *Klin. Wschr* 1963, 41, ?76–283

Selye, H., S. Grasso & N Padmanabhan. Protection by antimineralocorticoid against an otherwise fatal dihydrotachysterol intoxication. *Lancet* 1960, II, 1351.

Selye, H., B. Tuchweber & M. Jacqmin. Protection by various anabolic steroid against dihydrotachysterol induced calcinosis and catabolism. *Acta Endocr (Kbh.)* 1963, 49 589–602.

Selye, H. Catatoxic steroids. *Can. med Ass. J* 1969 101, 51–52.

Selye, H., M. Krajny & L. Savoie: Prevention of digitoxin poisoning by spironolactone. *Science* 1969 164, 842–843.

Siegel, S.. *Nonparametric statistics for the behavioral sciences.* In ● ● 1956, New York, McGraw-Hill Book Company Inc., 96–270.

Solymoss, B., H. G Clasen & S. Varga. Increased hepatic microsomal activity induced by spironolactone and other steroids. *Proc Soc exp Biol. Med (N Y.)* 1969 132, 940–942.

Wyngaarden, J B., H. L. Tiecke & M. H. Seevers: On the pharmacology of myanesin, with particular reference to its intramuscular administration. *Anesthesiology* 1949 10 529–543.

Acta pharmacol. et toxicol. 1970 28 149–160

From the Department of Pharmacology Queen's University Kingston, Ontario, Canada

Maximal Tolerated Daily Doses of Dicophane

By

E. M. Boyd and F. M. Liu

(Received November 18, 1969)

Abstract: Dicophane was given by intragastric cannula to young male albino rats in daily doses of from 43 to 250 mg/kg for 100 days or until 90 % of the animals had died. The LDO (100 days) or maximal dose which killed no animals when given daily for 100 days was 42.8 ± 1.6 mg/kg/day the LD50 (100 days) was 64.8 ± 1.4 mg/kg/day and the minimal LD100 (100 days) was 97.1 ± 2.8 mg/kg/day The 100-day LD50 index, or the LD50 (100 days) expressed as a percentage of the acute, oral LD50 (1 dose) was 17.6 ± 0.4. During the first 21 days of administration, the clinical signs of intoxication were similar to those recorded in studies on acute toxicity with single lethal doses. Clinical signs in 21 day-survivors were limited to polydipsia, diuresis, aciduria, and some loss of body weight. Autopsy at 100 days revealed degenerative changes in the kidneys, liver and testes, a stress reaction, hypertrophy of the liver and gastrointestinal organs, and capillary congestion and loss of weight (with variable changes in water content) of many body organs. The 100-day multiposal (subacute, chronic) toxicity of dicophane was similar to that of non-narcotic analgesics such as aspirin, phenacetin and paracetamol.

Key-words Toxicity – pesticides – DDT

The study reported in this communication was undertaken at the request of the World Health Organization in order to obtain information relative to the use of pesticides in countries in which the diet is low in protein. The initial approach to this problem was to determine the acute oral toxicity of a series of pesticides in albino rats fed for a suitable period of time on selected protein-deficient diets as compared with controls fed diets adequate in protein content. The results of these studies have been summarized by BOYD (1969). The second phase of the programme consisted of a comparison of the multiposal ("subacute chronic") toxicity of pesticides under similar dietary conditions. Multiposal toxicity was determined by methods which have been developed in this laboratory and reviewed by BOYD (1968a) The technique consists of

giving the agent by intragastric cannula once daily for 100 days and determining the syndrome of toxicity in the range of the LD50 (100 days) or daily dose which kills 50 / of animals over a period of 100 days of administration. An example of the application of this technique to a study of the multiposal toxicity of sucrose has been recently published by CONSTANTOPOULOS & BOYD (1968)

The clinico-pathological syndrome of toxicity to dicophane or DDT at the range of the oral LD50 (100 days) in albino rats will be described in this report as a reference standard for further similar studies on other pesticides and on rats fed protein-deficient diets Dicophane is the B P (1968) name for chlorophenothane (U S. P XVII) or DDT In an initial study on dicophane BOYD & DE CASTRO (1968) reported that the acute oral LD50 and associated clinico-pathologic syndrome of toxicity were essentially the same in albino rats fed for 28 days from weaning on a commercially-available diet containing 8 / of protein as casein as in animals fed 27 / dietary protein as casein and as in controls fed a standard laboratory chow The commercial production of the casein diets used by BOYD & DE CASTRO (1968) was discontinued shortly after their work was completed. The new commercially available low and normal protein diets contained different percentages of casein BOYD & DE CASTRO (1969) therefore undertook a study of the effects of feeding weanling rats diets containing from 0 to 81 / of casein. Dicophane was given intragastrically in a dose of 350 mg/kg body weight at the end of 4 weeks of dieting and killed 100 / of animals fed diets containing 0 and 3 / casein, 35 / of those fed 9 / casein, 12 / fed 27 / casein and 87 / of those fed 81 / casein. BOYD & KRIJNEN (1969) subsequently reported that the acute, oral LD50 of dicophane in rats fed 0 / casein for 28 days from weaning was 17 / of that in animals fed 27 / casein, when fed 3 / casein 34 / of that in rats fed 27 / 9 / casein 68 / and fed 81 / casein 27 / Relevant literature on dicophane is discussed in these several communications and in a recent review by DE CASTRO (1968)

Methods

The experiments were performed on 120 young male albino rats of a Wistar strain obtained from Woodlyn Farms, Limited, of Guelph, Ontario. The mean ± standard deviation initial body weight of the animals was 194 ± 12 g. They were housed in groups of 6 to 10 in boarding cages except during periods of clinical measurements when they were housed in metabolism cages, one rat per cage. The diet consisted of Rockland Rat Diet Complete obtained from Teklad, Incorporated, of Monmouth, Illinois, which was given ad libitum. This is a standard laboratory chow containing 24 % of protein, 56 % of carbohydrates, 8 % of ash, 4 % of fat, 5 % of fibre and adequate vitamins and minerals obtained by compounding foods such as soybean meal, fish meal, bone meal, dried whole milk, irradiated brewers' yeast, ground oats, ground wheat, ground corn, ground barley alfalfa meal and animal fat with added vitamins and minerals.

Dicophane was purchased from the Organic Chemicals Division of Eastman Kodak Company Rochester N Y as technical grade prepared to meet the specifications of the World Health Organization and of the British Pharmacopoeia, 1968. It was freshly dissolved in cottonseed oil, U S. P XVII, and administered intragastrically through a cannula attached to a syringe in a constant volume of 20 ml/kg body weight. The volume of solution was kept constant because of evidence, reviewed by BOYD (1968a), that variation in volume can affect the reaction to toxic doses of chemical agents given intragastrically

A pilot study was made t determine the difference between dosing the animals daily for 5 days a week as against 7 days a week at the same total weekly dose. A total weekly dose of 450 mg/kg was selected. One group received, therefore, 90 mg/kg/day for 5 days week (except Saturday and Sunday) and the second group received 64.3 mg/kg/day for 7 days a week. This pilot study was continued for 5 weeks at the end of which time the toxic reaction was found to be the same in both groups – for example, the death rate was 30 % at 5 weeks in both groups. In the definitive tests, therefore, the animals were given dicophane daily for 5 days a week.

The doses of dicophane given daily for 5 days a week were 0 (cottonseed oil alone) 60, 64, 90, 125 and 250 mg/kg. When given for one week or longer the daily dose given 5 days a week was multiplied by 5/7 to obtain the average daily dose used in calculating the LD50 (100 days). Dicophane administration was continued until 90 % of the rats had died or for 100 days, whichever occurred first.

At weekly intervals, or at shorter periods if indicated, clinical measurements were recorded on each rat placed singly in a metabolism cage for 24 hours. These measurements included body weight gain (or loss) per week in g, food intake in g/kg body weight per 24 hours, water intake in ml/kg/24 hours, colonic temperature in °F urinary volume in ml/kg/24 hours, urinary blood in units/kg/24 hours, urinary glucose and protein output in mg/kg/24 hours, urinary pH on 24 hour samples, and clinical signs semiquantitated in *clinical units of 1 to 4*.

Autopsy was performed on all rats which died and the gross pathology recorded. The histopathology was recorded of any tissue which appeared abnormal by gross examination and of all the tissues listed in table 2 in representative animals of each dosage group. The fresh wet weight of organs listed in table 3 was recorded in g on all survivors and controls at 100 days. The water content in the same survivors was measured in g water per 100 g dry weight of tissue on the organs listed in table 4 below

The results were analyzed statistically by application of t tests to differences between means and by regression of differences on dose of dicophane or interval after administration of dicophane. The LD50 was estimated at intervals of 7 days plotted against days of dosing, and the LD50 (100 days) calculated from analysis of the regression by the method of least squares

Details of method and of statistical analysis have been reported by CONSTANTOPOULOS & BOYD (1968) and reviewed by BOYD (1968a).

Results

The regression, on days of administration of dicophane, of values for the LD50 calculated at weekly intervals, was best fitted by the equation $Y = ((113-11 \log X) \times 0.71) \pm 1.4$ and from the equation the LD50 (100 days) ± S. E. was calculated to be 64.8 ± 1.4 mg/kg/day BUTLER *et al.* (1969) reported that a daily dose of 64 mg/kg body weight incorporated into the diet

Table 1

A summary of clinical signs of toxicity to daily administration of dicophane following the initial fortnight reaction.[a]

Sign	Daily dose of dicophane		
	Range of LD0 (100 days)	Range of LD50 (100 days)	Range of LD100 (100 days)
Weekly gain in body wt.: g	− 9	− 11	− 23
Food intake: g/kg/24 hours	+ 2	+ 6	+ 16
Water intake: ml/kg/24 hours	+ 10*	+ 12	+ 13
Colonic temperature: °F	− 0.1	− 0.4**	+ 0.6
Urinary volume: ml/kg/24 hours	+ 19*	+ 22	− 8
Urinary pH: 24 hour samples	− 12	− 9*	− 15

[a] The results are expressed as mean percent change from controls, given only cottonseed oil, during days 21 to 100. One asterisk indicates that the mean difference was significant at P = 0.05 to 0.02 and two asterisks at P = 0.01 or less.

of coho salmon killed 50 % of the fish in 90 days. The acute oral LD50 (1 dose) ± S. E. of dicophane in overnight-starved male rats has been recently found in this laboratory to be 368 ± 28 mg/kg (BOYD & DE CASTRO, 1968). The oral LD50 (100 days), therefore, was 17.6 ± 0.4 % of the acute oral LD50 (1 dose). Corresponding estimates of the maximal LD0 (100 days) were 42.8 ± 1.6 and of the minimal LD100 (100 days) 97.1 ± 2.8 mg/kg/day.

The clinical signs of toxicity to dicophane were most marked during the first two weeks and particularly during the first week of administration at all dose levels. The LD50 after 1 week was 79 mg/kg/day, after 2 weeks 70 mg/kg/day and from 28 to 100 days it remained at 65 mg/kg/day, i. e. there were no further deaths after the 4th week. The results will be described as those seen during the initial fortnight reaction and those recorded from 21 to 100 days.

In doses at the range of the maximal LD0 (100 days), the only clinical sign of toxicity during the initial fortnight reaction was diarrhea of mild intensity. There was a mild anorexia (average decrease in food intake was 11 %) accompanied by hypothermia, a doubling of urinary volume, proteinuria, glycosuria, aciduria, polydipsia and some loss of body weight. These signs, apart from hypothermia and aciduria, were found to be part of the syndrome of acute toxicity to dicophane by BOYD & DE CASTRO (1968). Signs remaining after 14 days are summarized in table 1 as the mean of measurements made from 21 to 100 days since there was no consistent change in these measurements with increasing duration of dicophane administration. Signific

ant signs remaining after the first fortnight were polydipsia, diuresis and aciduria at the LD0 (100 days)

An increase in the daily dose to the range of the LD50 (100 days) produced an initial fortnight reaction consisting of diarrhea, tremors, convulsions, ataxia, hyperkinesia, sialorrhea, hemodacryorrhea, and epistaxis associated with marked anorexia, loss of body weight, oligodipsia, hypothermia, glycosuria, proteinuria and aciduria. These initial signs were identical to those of acute toxicity reported by BOYD & DE CASTRO (1968), except that fever and alkalinuria occurred in the acute studies. The only signs seen in survivors after this initial fortnight reaction were polydipsia, hypothermia, diuresis and aciduria (table 1)

At the range of the minimal LD100 (100 days), the initial fortnight reaction was similar to that at the range of the LD50 (100 days) but each sign was more intense. In the few survivors of the initial fortnight reaction, there was a significant loss of body weight with an increased food and water intake, a slight but significant fever and an aciduria (table 1)

Table 2

Histopathological changes observed at autopsy on albino rats given dicophan daily for 100 days.

Organ	Histopathology
Adrenal glands	Lipoid droplets prominent in the zona fasciculata.
Brain	Occasionally minor capillary congestion of the meninges.
Gastrointestinal tract.	
Cardiac stomach	Normal appearance.
Pyloric stomach	Normal appearance
Small bowel	Villi hypertrophied.
Caecum	Normal appearance.
Colon	Normal appearance.
Heart	Occasionally mild capillary congestion of the myocardium.
Kidneys	Minor capillary congestion of the loop region, occasionally minor cloudy swelling of the tubules
Liver	Sinusoidal congestion and varying degrees of fatty degeneration.
Lungs	Minor capillary congestion.
Muscle (ventral abdominal wall)	Normal appearance.
Salivary (submaxillary) glands	Ducts of serous glands dilated with secretion.
Skin	Normal appearance.
Spleen	Red pulp contracted.
Testes	Inhibition of spermatogenesis varying from mild at 64 mg/kg/day to complete at 89 mg/kg/day
Thymus gland	Loss of thymocytes, increasing with increase in daily dose of dicophane.



Fig. 1. A photomicrograph of the testis of a rat after oral administration of 89 mg of dicophane per kg body weight per day for 100 days showing atrophy of spermatogenic tissue and oedema and hypertrophy of the interstitial tissue (stain: hematoxylin-phloxine-saffron, magnification × 100).

A summary of the pathological lesions observed at autopsy is presented in table 2. In contrast to the acute gastroenteritis reported from single oral lethal doses by BOYD & DE CASTRO (1968), the gastrointestinal tract appeared to adapt to the daily presence of large amounts of dicophane and, if anything, the mucosa appeared to be hypertrophied. There was mild vascular congestion of several body organs including the meninges, myocardium, liver, kidneys and lungs. There was a stress reaction in the adrenal thymus gland and spleen. Degenerative changes were present in the kidneys, liver and testes. Spermatogenic tissue was almost completely absent in rats given 89 mg of

dicophane per kg body weight per day for 100 days as shown in fig. 1 Inhibition of spermatogeneais may be related to the suggestion of WELCH, LEVIN & CONNEY (1969) that appreciable amounts of oestrogenic metabolites may be formed from large doses of dicophane in rats.

Table 3

Changes in the wet weight of body organs of albino rats after 100 days of daily administration of dicophane.¹)

Organ	Average daily dose of dicophane (mg/kg)		
	43 (N = 19 plus 10 controls)	64 (N = 13 plus 10 controls)	89[*]) (N = 2 plus 10 controls)
Adrenal glands	+ 16 6	+ 30.9**	+ 26.0
Brain	− 0.3	+ 0.8	− 7.1
Gastrointestinal tract.			
Cardiac stomach	+ 15.8**	+ 10.2	− 10.2
Pyloric stomach	+ 3.7	+ 2.8	+ 2.2
Small bowel	+ 16.5**	+ 3.6	+ 18.2
Caecum	+ 25.3**	+ 0.2	+ 36.3
Colon	+ 11.2*	− 3 3	− 12.0
Heart	− 9.2*	− 0.7	− 20.1
Kidneys	− 2.8	− 10.6	− 12.9
Liver	+ 15.8*	+ 22.2*	+ 37 7
Lungs	+ 3.8	+ 6 1	+ 6.0
Muscle (ventral abdominal wall)	− 14.0*	− 12.2*	− 16.3
Salivary (submaxillary) glands	+ 0.2	− 3.1	− 7.0
Skin	− 20.5**	− 22.4**	− 28.7
Spleen	− 10.8*	− 0.7	− 14.2
Testes	+ 6.2	+ 12.2	− 59.2
Thymus gland	− 32.8**	− 22.0*	− 86 7
Residual carcass	− 12.5**	− 15.2	− 26.4
Autopsy body weight	− 12.6**	− 14.3**	− 25 7

¹) Wet weight was measured in grams. The results are expressed as the mean percent change from controls given cottonseed oil, specifically as $((\bar{X}_d - \bar{X}_c) / \bar{X}_c) \times 100$ where $\bar{X}_d$ is the mean in dicophane-treated animals and $\bar{X}_c$ in controls. One asterisk indicates that $\bar{X}_d - \bar{X}_c$ was significant at P = 0.05 to 0.02 and two asterisks at P = 0.01 or less.
[*]) Statistical calculations of probability omitted because of low value of N

Shifts in the fresh wet weight of body organs of rats surviving to 100 days are summarized in table 3 Increasing daily doses of dicophane produced increasing loss of body weight mainly due to loss of weight in skeletal muscle and skin. There were no significant changes in the weight of the brain, pyloric stomach, and salivary glands while the weight of the adrenal glands, liver

parts of the gastrointestinal tract and occasionally lungs was increased. Increase in the weight of the liver may indicate increased ability of the survivors to detoxify dicophane the increase was greater the higher the daily dose of dicophane. The only other change which was consistently related to the daily dose of dicophane was increasing loss of weight in organs such as kidneys, skin and residual carcass. The weight of the testes was decreased by the highest daily dose of dicophane but was unaffected by the lowest daily dose, thus confirming the results of OTTOBONI (1969) who reported no effect on reproduction from daily doses of the order of 10–20 mg/kg added to the diet of rats.

Table 4

Changes in the water content of body organs of albino rats after 100 days of daily administration of dicophane.)

Organ	Average daily dose of dicophane (mg/kg)		
	43 (N = 19 plus 10 controls)	64 (N = 13 plus 10 controls)	89b) (N = 2 plus 10 controls)
Adrenal glands	− 9.1	− 15.0*	− 26.9
Brain	− 0.2	− 3.2	− 0.2
Gastrointestinal tract:			
Cardiac stomach	− 0.7	+ 16.5	− 30.0
Pyloric stomach	− 1.2	+ 7.9*	− 25.7
Small bowel	+ 3.2	+ 8.0*	− 0.2
Caecum	− 6.3	+ 5.2	− 19.3
Colon	− 1.8	+ 15.3**	− 0.7
Heart	+ 1.7	+ 5.4	− 0.2
Kidneys	− 0.5	+ 4.2*	− 3.9
Liver	− 5.2	− 6.8*	− 0.1
Lungs	− 0.9	+ 8.1	− 15.3
Muscle (ventral abdominal wall)	+ 3.4	+ 4.4	− 0.1
Salivary (submaxillary) glands	− 5.9	+ 3.2	− 13.3
Skin	+ 10.2*	+ 28.3**	− 0.6
Spleen	− 0.8	− 0.6	− 0.3
Testes	0.0	− 0.4	− 43.0
Thymus gland	+ 3.3	+ 18.8	− 8.8
Residual carcass	+ 2.8	+ 8.3	− 0.2

a) Water content was measured as grams water per 100 grams dry weight of tissue. The result are expressed as mean percent change from controls given cottonseed oil, specifically as $((\bar{X}_d - \bar{X}_c) / \bar{X}_c) \times 100$ where $\bar{X}_d$ is the mean in dicophane-treated rats and $\bar{X}_c$ in controls. One asterisk indicates that $X_d - X_c$ was significant at P = 0.05 to 0.02 and two asterisks at P = 0.01 or less.

b) Statistical calculations of probability omitted because of low value of N

Daily doses of dicophane in the range of the maximal LD0 (100 days) produced few significant changes in organ water contents as indicated by results presented in table 4 There was some tendency to dehydration in the adrenal glands and to hydration in the skin.

As the daily dose reached the LD50 (100 days) hydration appeared in many organs of the survivors. As noted in table 4 this was particularly true of most parts of the gastrointestinal tract, the skin and *thymus gland*. The adrenal glands and liver continued to exhibit dehydration and water levels were insignificantly altered in certain organs such as the brain, muscle salivary glands, spleen and testes.

In two survivors of a daily dose of 89 mg/kg, the mean water levels at 100 days were lowered in all organs, particularly in the adrenal glands, most parts of the gastrointestinal tract, the lungs, salivary glands, and testes, as shown in table 4

Discussion

The results of this study demonstrated that albino rats rapidly develop tolerance to the lethal effects of orally administered dicophane. During the first three weeks of administration of daily doses ranging from 43 to 250 mg/kg or from 12 to 68 / of the acute oral LD50 the syndrome of toxicity was similar to that recorded in acute or single lethal dose studies by BOYD & DE CASTRO (1968). Doses over 43 mg/kg/day produced deaths during but not after the initial three weeks. Survivors of this initial 3 week-reaction lived to 100 days with continuing daily administration of dicophane During the period of 21 to 100 days they had some loss of body weight, polydipsia, diuresis, aciduria and minor changes in body temperature. Autopsy at 100 days revealed more or less dose-dependent degenerative changes in the kidneys, liver and testes, a stress reaction, hypertrophy of the liver and gastrointestinal tract and varying degrees of capillary congestion and loss of weight (in spite of an unaltered or augmented food intake) of many body organs.

Many clinico-pathological features of the reaction of rats to 100 days of oral administration of dicophane were the same as those reported in similar 100 day-studies with other chemical agents. A distinguishing feature in each case is the daily dose which produces the toxicity syndrome. A review of available data disclosed that while the LD50 (100 days) varies widely (over four hundred fold) it is much less variable when expressed as a percentage of the acute LD50 (1 dose) The quotient is termed the 100-day LD50 index and available values for this index are assembled in table 5 The 100-day LD50 index is an estimate of 100-day chronic toxicity in one single figure. The higher the index the greater is the proportion of an acute LD50 (1 dose) which can be tolerated by daily administration for 100 days and therefore the

Table 5

The 100-day LD50 index of eleven agents calculated from available evidence.

Agent	Species	Route of Administration	100-day LD50 index ± S. E.	Reference
Phenacetin	Guinea pig	Oral	12.3 ± 4.3	BOYD & CIAMPI (1969)
Atropine	Rabbit	Intramuscular	13.2 ± 0.8	BOYD & BOYD (1962)
Aspirin	Rat	Oral	16.4 ± 0.5	BOYD (1968b)
Pilocarpine	Rat	Oral	17.1 ± 2.2	BOYD & JARZYLO (1968)
Dicophane	Rat	Oral	17.6 ± 0.4	This paper
Paracetamol	Rat	Oral	20.6 ± 0.5	BOYD & HOGAN (1968)
Phenacetin	Rat	Oral	27.1 ± 0.5	BOYD & HOTTENROTH (1968)
Benzylpenicillin	Rat	Oral	61.8 ± 3.0	BOYD & SELBY (1962)
Sodium chloride	Rat	Oral	71.7 ± 3.2	BOYD, ABEL & KNIGHT (1966)
Caffeine	Rat	Oral	78.2 ± 1.6	BOYD, DOLMAN, KNIGHT & SHEPPARD (1965)
Sucrose	Rat	Oral	80.6 ± 3.7	CONSTANTOPOULOS & BOYD (1968)

greater is the relative safety of the agent under study. Conversely, the lower the value of the 100-day LD50 index the relatively more toxic is the agent for repeated daily administration. Further information on the 100-day LD50 index and on other indices of chronic toxicity has been recently reviewed by BOYD & CIAMPI (1969).

It will be noted from data in table 5 that the 100-day LD50 index is low for agents such as atropine, pilocarpine and dicophane which might well be expected to be relatively toxic when given daily in large amounts. For agents such as sodium chloride, caffeine and sucrose, which are part of the daily diet of most people the 100-day LD50 index is high, indicating relative safety on chronic use as again might be anticipated. The widely used analgesic drugs aspirin, phenacetin, and paracetamol have a relatively low 100-day LD50 index. By referring to discussions on these analgesics in references cited in table 5 it may be concluded that if they are used in doses not exceeding those in government-approved labels (i. e. one or two tablets three or four times a day for a limited period of time) little or no toxicity would be anticipated

from these studies on rats. If these analgesics are abused and taken in amounts of the order of 25 to 100 tablets daily for a period approximating one tenth of the expected lifespan (corresponding to 100 days in rats), the data on rats suggest they would produce toxic effects as have been reported in man. Abuse of these analgesics is possible when they can be obtained without a physician's prescription and "over-the-counter" or OTC" The incidence of abuse of OTC analgesics has been estimated by BOYD (1968b) to be of about the same order in Canada as the incidence of narcotic addiction.

The results summarized in table 5 differ from those in recent studies by WEIL *et al.* (1969) who found a rather constant relationship between minimal effect levels of some 20 materials added to the diet of rats for periods of 7 and 90 days. The main criterion of a minimal effect was loss of body weight food intake was not reported. In our studies the material was given daily by intragastric cannula and the food intake was usually not significantly affected until the daily dose exceeded the LD50 (100 days) though growth rate was usually depressed. On the other hand, the results in table 5 are somewhat similar to those of HAYES (1967) who added drugs to the diet of rats and then divided the LD50 (1 dose) by the daily dose which killed 50 / of rats at 90 days in order to obtain a chronicity factor" which is basically a reciprocal of the 100-day LD50 index.

Acknowledgement

This project was assisted by Grant F5/181/5 of the World Health Organization.

REFERENCES

Boyd, C. E. & E. M. Boyd: The chronic toxicity of atropine administered intramuscularly to rabbits. *Toxicol. Appl. Pharmacol.* 1962, 4 457–467

Boyd, E. M.. Predictive drug toxicity assessment of drug safety before human use. *Can. Med. Assoc.* J 1968a, 98, 278–293.

Boyd, E. M.. Analgesic abuse: maximal tolerated daily doses of acetylsalicyclic acid. *Can. Med. Assoc. J* 1968b, 99 790–798.

Boyd, E. M.: Dietary protein and pesticide toxicity in male weanling rats. *B ll. Wld. Hlth. Org* 1969 40 801–811.

Boyd, E. M., M. M. Abel & L. M Knight. The chronic oral toxicity of sodium chloride at the range of the LD50 (0.1L). *Can. J Physiol. Pharmacol.* 1966, 44, 157–172.

Boyd, E. M. & O C. Clampt: The oral 100-day LD50 index f phenacetin in guinea pigs. *Toxicol. Appl. Pharmacol.* 1970 16, 1 7

Boyd, E. M. & E. S. De Castro: Protein-deficient diet and DDT toxicity *B ll. Wld. Hlth. Org* 1968, 38, 141 150.

Boyd, E. M. & E. S. De Castro: The toxicity of dicophane (DDT) in relation to dietary protein intake. *Indust. Med. Surg* 1970, in press.

Boyd, E. M., M. Dolman, L. M. Knight & E. P. Sheppard. The chronic oral toxicity of caffeine. *Can. J. Physiol. Pharmacol.* 1965. 43, 995–1007

Boyd, E. M. & S. E. Hogan. The chronic oral toxicity of paracetamol at the range of the LD50 (100 days). *Can. J. Physiol. Pharmacol.* 1968, 46, 239–245.

Boyd, E. M. & S. M. H. Hottenroth. The toxicity of phenacetin at the range of the oral LD50 (100 days) in albino rats. *Toxicol. Appl. Pharmacol.* 1968, 12, 80–93.

Boyd, E. M. & S. V. Jarzylo. Daily oral doses of pilocarpine tolerated by albino rats. *Arch. Int. Pharmacodyn.* 1968, 174, 84–98.

Boyd, E. M. & C. J. Krijnen. Dietary protein and DDT toxicity. *Bull. Environ. Contam. Toxicol.* 1969. 4. 256–261.

Boyd, E. M. & M. J. Selby. The chronic oral toxicity of benzylpenicillin. *Antib. and Chemotherap.* 1962, 12, 249–262.

Buhler. D. R., M. E. Rasmusson & W. E. Shanks. Chronic oral DDT toxicity in juvenile coho and chinook salmon. *Toxicol. Appl. Pharmacol.* 1969. 14. 535–555.

Constantopoulos, G. & E. M. Boyd. Maximal tolerated amounts of sucrose given by daily intragastric administration to albino rats. *Fd. Cosmet. Toxicol.* 1968, 6, 717–727

De Castro, E. S.: *Protein deficient diet and dicophane toxicity in rats.* Graduate Thesis. Douglas Library. Queen's University. Kingston, Ontario, Canada, 1968.

Hayes, Jr. W. J. The 90-dose LD50 and a chronicity factor as measures of toxicity. *Toxicol. Appl. Pharmacol.* 1967. 11. 327–335.

Ottoboni, A. Effect of DDT on reproduction in the rat. *Toxicol. Appl. Pharmacol.* 1969. 14. 74–81.

Weil. C. S., M. D. Woodside, J. R. Bernhard & C. R. Carpenter. Relationship between single-peroral, one-week and ninety-day rat feeding studies. *Toxicol. Appl. Pharmacol.* 1969. 14, 426–431.

Welch, R. M. W. Levin & A. H. Conney. Estrogenic action of DDT and its analogs. *Toxicol. Appl. Pharmacol.* 1969. 14, 358–367

Acta pharmacol. et toxicol 1970 **28** 161–176.

From the Department of Pharmacology Karolinska Institutet,
Stockholm, Sweden

Emetic and Gastric Secretory Stimulating Action by Esters of some Amino Acids

By

Carl-Eric Elwin

(Received October 13, 1969)

Abstract. Esters of amino acids reduce gastric acidity following intragastric administration but simultaneously exert an emetic effect. A series of experiments was designed on a total of 13 dogs to elucidate the mechanisms of the effects of ethyl-ester of glycine, diethyl-ester of glutamic acid, diisopropyl-ester of glutamic acid, isopropyl-ester of phenylalanine and isobutyl-ester of valine. Oral administration or injection of the esters by means of gastric cannula with free passage to the duodenum induced emesis in 17 of 23 experiments (74 %) on 6 dogs in an average time of 1[illegible] minutes. Occlusion of the duodenum with a Foley catheter distal to the pylorus prevented an emetic response during gastric administration. Emesis was [illegible]uced in 47/57 administrations (82 %) on 10 dogs 0–10 cm distal to the pylorus by emetic threshold doses within an average time of 8 minutes. Emetic effect declined with distance from the pylorus and below 50 cm no emetic response occurred. The emetic threshold dose in general was 2–4 ml of the esters except for diethyl-ester of glutamic acid, the threshold dose of which was 0.2–0.5 ml. A refractory period of 1 1½ hour's duration to an emetic dose was observed. Hydrochlorides of esters tested showed a similar emetic pattern. I control experiments, copper sulphate induced vomiting from both the stomach and the duodenum in amounts indicating more potent emetic action (0.025–0.25 g). Diethyl-ester of glutamic acid was also administered intravenously but amounts 2–6 times larger than the duodenal emetic dose did not evoke emesis. Blockade of the emetic effect was demonstrated by 0.4–2.0 % lidocaine (10–30 ml) and by 0.5–2.0 mg atropine administered locally in the duodenum, whereas the subcutaneous administration of atropine showed a less pronounced blocking effect. Esters of amino acids may act as local irritants. – Gastric administration of 1 2 ml of ester raised the pH to 7.0–7.6 immediately and this lasted for 5–15 min. Neither gastric nor duodenal administration increased the volume or total acid output of innervated or vagally denervated fundic pouches. Perfusion of the isolated, innervated antrum pouch, however caused an increase of total acid output of the fundic pouch, indicating a release of the antral stimulatory hormone, gastrin.

Key-words. Animal experiments – atropine/pharmacodynamics – copper sulphate/pharmacodynamics – duodenum/drug effects – esters/pharmacodynamics gastrin – gastric juice/secretion – vomiting/chemically induced.

The emetic action produced by many agents, can be initiated from peripheral as well as central sites and for some strong emetic agents a simultaneous peripheral and central mechanism of action has been demonstrated (BORISON & WANG 1953) A peripheral emetic action may be induced from almost any site in the body Agents causing gastrointestinal irritation very often induce emesis and the duodenal mucosa has been shown to be more sensitive to such stimuli than the gastric mucosa. The nervous impulses to the emetic center may pass by way of afferent fibres in either the vagal or the sympathetic nerves (BORISON & WANG 1953).

It has been reported that some aliphatic alcohols stimulate gastric acid secretion following administration into the stomach. The mechanism of action seems to be solely dependent on an antral humoral factor – gastrin – and a relationship has been shown between the stimulating activity and molecular configuration (ELWIN 1969) Some mono amino acids also stimulate gastric acid secretion on administration into the antrum (ELWIN unpublished).

Since they are amphoteric compounds, the esters of amino acids reduce gastric acidity In a previous study of this question it was observed that some of the compounds exerted a marked emetic effect (ANDERSSON & ELWIN 1957) Preliminary data then indicated that the isopropyl-ester of phenylalanine, the isobutyl-ester of valine and the diisopropyl-ester of glutamic acid induced vomiting during administration into the proximal part of the duodenum. The emetic effect of these esters was confirmed in the present study and two additional esters, ethylester of glycine and diethyl-ester of glutamic acid were investigated. As some aliphatic alcohols (e. g. ethanol) and some mono amino acids (e. g. glycine) stimulate gastric acid secretion it was also thought to be of interest to include in this study the effect of the esters on gastric function. Experiments were performed in order to localize within the gastrointestinal tract the area sensitive to the emetic effect of the agents, and also to study the influence of some of the esters on gastric acid secretion. With one of the esters some experiments were done to find out whether the emetic effect was of local or central origin.

Methods

Surgical procedure

The experiments were performed on 13 dogs weighing 10–19 kg. A variety of surgical approaches were used in order to localize the area within the gastrointestinal tract, sensitive to the emetic action(table 1) Duodenal cannulas were inserted 2–5 cm distal to the pylorus and gastric cannulas were inserted into the corpus region of the stomach well off the antrum-corpus boundary to avoid mechanical stimulation of the antrum.

In order to register in addition the effect on gastric acid secretion 9 dogs with vagally denervated pouches of the Heidenhain type or vagally innervated pouches of

Table 1

Surgery performed in dogs to allow administration of the various esters in different parts of the stomach and the duodenum.

Dog No.	Cannulas		Antrum pouch	Fundic pouch H = Heidenhain type P = Pavlov type
	Gastric	Duodenal		
36		x	1)	P
39		x	1)	P
56	x			P
71		x	x	P
83	x	2) x		P
87		x	2) x	
88	x	x		
89				
90		x		P
95		x		
141		x	x	P
179			x	H
180		x		H

1) Antrectomized
2) Mann-Bollman fistula

the Pavlov type were used. Two dogs were provided with a Mann-Bollman fistula between the duodenum (dog 83) or the isolated antrum (dog 87) and the body surface. The fistula, as intestinal retroperistaltic anastomosis was prepared surgically from the distal part of ileum and the end-to-side anastomosis located about 10 cm from the pylorus (dog 83).

Four dogs had an isolated innervated antrum pouch as described by UVNÄS *et al* 1956. In three of these dogs the pyloric end of the antrum was brought through the abdominal wall as a cutaneous fistula and in the fourth dog the isolated antrum pouch was connected with the abdominal surface by a Mann-Bollman fistula. Test agents could be administered directly into the antral pouches. In two dogs with fundic pouches and duodenal cannulas, the antrum had been removed because these dogs as well as most of the others were also used in other experimental series.

Test procedures.

The animals were fasted for about 18 hours before the experiments were started. In dogs provided with fundic pouches, the basal acid secretion was recorded for at least one hour before the administration of the test solutions. Gastric acid output was collected in 15-min. portions. The volume was measured and the amount of free and total acid determined. Each portion was titrated with 0.01 N-NaOH, using a mixture of Töpfer' reagent and phenolphthalein as indicator. When emesis and retching are discussed in this paper they are defined in the same way as that described by BORISON & WANG 1953. The term emesis is applied to expulsion of gastrointestinal contents through the mouth. Retching is defined as a labored rhythmic activity of the respiratory musculature preceding vomiting.

0.2 16 ml of the esters were administered as single doses in saline corresponding to 2 ml ester per 10 ml NaCl. The following routes of administration were used, oral (or via gastric fistula), antral (instillation or perfusion), intraduodenal, intrajejunal and intravenous. The intestinal lumen was occluded by the introduction of a Foley catheter through the duodenal cannula and inflating 10–20 ml of air into the balloon. The volume of air used in the individual dog was to a certain extent related to the size of the intestine and was determined in control experiments in the following way The Foley catheter was placed in the duodenum and the balloon inflated with 10, 20, 30 ml etc. of air until the pressure of the balloon against the intestinal wall evoked an emetic response. During the experiments, the balloon was filled with a volume of air below th threshold volume for an emetic response. This volume of air never caused vomiting in the control experiments. The esters could be given into the stomach or at different levels within the duodenum in front of or behind the balloon. A Miller-Abbott tube was used for administration further down the duodenum.

The ethyl-ester of glycine and the diethyl-ester of glutamic acid were also administered intravenously The diethyl-ester of glutamic acid was the only ester sufficiently solubl in water to be given intravenously whereas the other esters had a low water and fat solubility To overcome this, the solution of ethyl-ester of glycine in saline was emulsified in a small manual homogenator before administration.

I control experiments, 10–20 ml of saline only the corresponding alcohol or amino acid, or the ester hydrochloride were tested separately

The effect of ethyl-ester of acetic acid and of diethyl-ester of glutaric acid was also investigated.

Copper sulphate (0.5 % 5–20 ml) was administered in some experiments as an agent known to have a peripheral emetic action, probably by causing gastrointestinal irritation.

Blockade of the emetic action was attempted by the use of atropine given locally (0.5 1.0 and 2.0 mg) or subcutaneously (0.5 1.0 and 2.0 mg), and by the use of a local anaesthetic, lidocaine, xylocain® (lidocainum NFN) Astra (0.4 or 0.5 % 10–30 ml) For intraduodenal administration atropine was given in 10, 20 or 30 ml saline.

In situ measurements of pH of the stomach content during administration of the esters were performed in some experiments. A Beckman glass electrode, 4 mm in diameter specially designed for measurements in the gastrointestinal tract was used. The intraluminal reference electrode was a potassium chloride-agar-filled open tipped polyethylene tube connected to a calomel electrode. The glass electrode and the reference lead were connected to a pH meter (Radiometer, Denmark). The open tip of the reference electrode and the glass electrode were tied together in such a way as to allow location 2 3 cm apart in situ. The pH electrode was standardized before and after each test against standard buffers.

Results

Emetic action during administration in the stomach and in the duodenum.

A. *Gastric administration* (table 2)

The emesis producing effect of diisopropyl-ester of glutamic acid, isopropyl-ester of phenylalanine and isobutyl-ester of valine during stomach application was studied in 6 dogs with free passage to the duodenum. In each experiment an emetic threshold dose of the test agent was administered from 1 up to 8

Table 2

Emesis by esters of amino acids during application in the stomach with free and occluded passage to the duodenum and during application into the isolated antral pouch. Figures in brackets indicate administration of ester hydrochlorides.

Dog No.	Free duodenal passage				Occluded duodenal passage				Isolated antral pouch	
	Orally		Gastric fistula		Orally		Gastric fistula			
	Expts.	Em.	Expts.	Em.	Expts.	Em.	Expts.	Em.	Expts.	Em.
56			4(2)	4(2)						
71	1	1							2	0
83			1	1						
87									3	0
88			10(1)	6(0)			3	0		
89	1	1			3	0				
90	3	2								
141									(4)	(1)
179									3(2)	1(0)

times. No difference in effect was observed whether the agents were administered orally or by injection through the gastric cannula. Emesis was obtained in 17 of 23 experiments (74 /). The occurrence of the emetic response ranged from 5 to 30 minutes, the average being 11 minutes.

No emesis occurred by oral or by direct gastric administration in experiments where the gastrointestinal passage was occluded distal to the pylorus by the airfilled balloon of a Foley catheter. In 4 dogs with an isolated, innervated antrum pouch, 2–16 ml of the ester or the hydrochloride were either instilled into or perfused through the antrum. The hydrochloride was tried since the esters are transformed to that compound when they come into contact with the acid gastric content during administration into the main stomach. Ethyl-ester of glycine, diisopropyl-ester of glutamic acid and isopropyl-ester of phenylalanine were investigated. Emesis occurred once in a total of 8 experiments (12.5 /) with the actual esters, and once in 6 experiments with the corresponding hydrochlorides (16.6 /).

B. *Duodenal administration* (table 3 and 4).

In 70 experiments on 10 dogs the esters were given directly into the proximal part of the duodenum (0–10 cm from the pylorus) through a short rubber tube entering via the duodenal cannula. The number of administrations varied between 1 and 3 in each experiment. Administrations were done with the ethyl-ester of glycine, isobutyl-ester of valine, isopropyl-ester of phenylalanine and diisopropyl – or diethyl-ester of glutamic acid. In table 3 have been listed

Table 3

Emesis by esters of amino acids during application into the duodenum 0–10 cm from the pylorus. Figures in brackets indicate administration of ester hydrochlorides.
Em. = emesis.
Adm. = number of administrations.

Dog No.	Duodenum (0–10 cm from the pylorus)	Isobutyl-valine		Isopropyl-phenylalanine		Diisopropyl-glutamic acid	
	Expts.	dose ml	Em./adm.	dose ml	Em./adm.	dose ml	Em./adm.
36	2			1	0/1	5	0/1
39	2			2	1/1	5	1/1
71	2			2	1/2		
87	2					2	2/2
88	8	2	1/1	2	0/2	4	2/2
				4	2/3		
89	9(3)	2	4/4	2	2(1)/3(2)	2	2(1)/2(1)
90	3	2	2/2	2	1/1		
95	7	2	0/1	2	1/1		
		4	1/2	4	2(1)/2(1)		
		Ethyl-glycine				Diethyl-glutamic acid	
141	11	3–6	0/5	1	0/1	0.5	1/3
		7	1/2			0.7	0/2
						1.0	1/1
180	13(8)	2	0/1	1–2	0/3	0.2	3/3
		3	2/2	3	1/1	0.5	3/3
		4	0(3)/4				
		5	1(2)/4				
		6	(0)/1				
		7	1/1				

emetic effects which occurred after various doses in relation to the number of administrations according to the following criteria. The first administration of the day in the duodenum of the ester under study was always included as well as the repeated administration 2 to 3 hours later of a larger dose of the same ester or the administration of another ester. Careful choice of the time interval was regarded as important to prevent interference of the refractory period (see below) with the results. No administrations in connection with the use of blocking agents were included except when the ester was given before the blocking agent. Various doses were given to find out the emetic threshold dose of each agent. A dose of 3 ml of ethyl-ester of glycine initiated vomiting in one of two dogs tested but as much as 7 ml was required in the other dog. 2 ml of isobutyl-ester of valine regularly provoked emesis in three of 4 dogs.

Table 4

Emesis by esters of amino acids in the duodenum at various distances from the pylorus.

Dog No.	Distance from pylorus in cm: 0 – 10		10 – 20		20 – 30		30 – 40		40 – 50		50 – 60		60 – 70		70 – 80		80 – 90	
	Number of Expts.	Em.	Number of Expts.	Em.	Number of Expts.	Em.	Number of Expts.	Em.	Number of Expts.	Em.	Number of Expts.	Em.	Number of Expts.	Em.	Number of Expts.	Em.	Number of Expts.	Em.
88	9	6	2	0	5	1	2	0	4	1			1	0	1	0		
89	10	9	5	3	2	2	5	2	5	2	2	0	1	0				
95	8	6			1	0	2	2	3	2	1	0	2	0			1	0

In the fourth dog (95) 2 ml caused retching only whereas 4 ml was the emetic dose. 1 ml of isopropyl-ester of phenylalanine was ineffective in 3 of 9 dogs tested but vomiting occurred in 5 of 7 dogs with 2 ml. 2 ml of diisopropyl-ester of glutamic acid was found to be the effective emetic dose in 2 of 5 dogs.

0.2 or 0.5 ml of diethyl-ester of glutamic acid caused vomiting in one dog in every experiment and in another dog 0.5–1.0 ml of this ester apparently represented the emetic threshold dose. The emetic action of this ester seems to be slightly more pronounced when compared with doses of other esters. Unfortunately the ester was not available for experimental use at the same time as the diisopropyl-ester of glutamic acid and a comparison of the effect of these two esters on the same dogs could therefore not be done.

Table 4 shows the emetic action of the esters when administered at various distances from the pylorus. Actually the distance was measured from the opening of the cannula or the Mann-Bollman fistula but can be approximated to correspond to the pylorus. Emesis is most regularly induced from the upper part of the duodenum 0–10 cm from the pylorus. The mucosa at 10–50 cm distance from the pylorus was slightly less sensitive and below 50 cm from the pylorus no emetic response occurred. Application of esters at that distance or further down the duodenum tended to cause defaecation and blood-stained diarrhoea in one dog as well as retching, indicating local mucosal irritation.

After duodenal administration vomiting occurred within an average of 8 minutes with a range of from 2 to 30 minutes. An emetic response resulted in refractory condition to a second dose. In 22 experiments on three dogs (88 89 95) another administration within 30–60 min. with the same amount of the ester caused emesis only twice in 10 experiments on dog 89 and twice in 9 experiments on dog 95 After 80–100 minutes vomiting occurred more frequently (3/6 experiments and 1/2 experiments respectively). In dog 89 administrations after 130–150 minutes initiated a second emetic response in 2 of 3 experiments. Thus, there seems to be a refractory period of 1–1½ hours duration.

The esters undergo transformation to hydrochlorides in the stomach and these compounds might be responsible for the emetic effect after transport into the upper part of the duodenum. 2 ml of the hydrochloride of isopropyl-ester of phenylalanine administered directly into the stomach of dog 56 caused emesis in 2/2 experiments within the same latent period as the ester itself. 4 or 5 ml of the hydrochloride of ethyl-ester of glycine showed the same pattern as the ester itself in 5 experiments on dog 180 A few experiments of this type were undertaken on dogs 89 and 95 and confirmed the above findings (see table 3).

A number of control experiments using corresponding amounts of saline,

isopropyl alcohol, glutamic acid and phenylalanine were performed in four of the dogs. An emetic response never occurred with these test agents in the amounts used.

Copper sulphate is known to cause vomiting when it comes into contact with the upper part of the gastrointestinal tract. In three dogs (87 89 95) the emetic potency of this agent was compared with that of the esters. Emesis was already induced from the stomach with copper sulphate. Vomiting oc curred during antrum perfusion (dog 87 1 experiment) and after oral administration (dogs 89 95 2 experiments) with the duodenum passage occluded distal to the pylorus by a balloon. Perfusion of the isolated antrum pouch for 10 min. with 50 ml of a 0.5 / $CuSO_4$ solution (= 0.25 g $CuSO_4$) caused repeated emetic responses starting after 5 min. Five ml 0.5 / $CuSO_4$ given orally produced emesis in dog 89 but amounts up to 20 ml did not cause an emetic effect in dog 95 Administrations were also done directly through the cannula in the upper part of the duodenum on these dogs and emesis was provoked by 5 ml of the copper solution (= 0 025 g) in dog 89 and by 20 ml of the solution (= 0 1 g) in dog 95 5 10 and 12 ml showed no effect in this dog but with 15 ml retching occurred frequently

Comparison of emetic action after duodenal and intravenous administrations and of effects of esters of amino acids and the corresponding carboxylic acids

Some experiments were performed with intravenous administration of the ethyl-ester of glycine and the diethyl-ester of glutamic acid (dogs 141 and 180) to indicate whether the emetic effect also included a central trigger mechanism. Just a few experiments were done with the ester of glycine because shortly after the start of the intravenous administration the dogs became anxious and seemed to suffer from itching, oral oedema, a copious salivary secretion, increased respiratory rate and retching. Doses varied be tween 0.3 and 6.0 ml. Emesis occurred in doses similar to those which exert an emetic action when given locally into the duodenum However doubts about the pure emetic nature of the response made it hard to draw any conclusions about a central emetic action. Diethyl-ester of glutamic acid was administered intravenously in dog 180 only In 10 administrations during 7 experiments with doses increasing from 0.1 to 1.2 ml emesis or signs of retching did not occur

Emetic responses from the duodenum by these esters were also compared with the effect of esters of the corresponding carboxylic acids, acetic acid and glutaric acid. Ethyl acetate was administered through the duodenal fistula of dog 141 6 times in 6 experiments in doses of 1, 2, and 5 g (= 1 1 2.2 and 5.5 ml respectively) and 14 times in 11 experiments on dog 180 in doses varying between 1 and 7 g. Vomiting already occurred at the 1 g dose level in dog 141 (1/2 experiments) and regularly with larger amounts. The time

Table 5

Blockade of emetic effect of esters of amino acids by local or subcutaneous administration of lidocaine and atropine. In the control ex periments some figures have been left out to avoid repetition and can be found above for that dog.

Blocking Agent Dose, volume, route of administration	Dog No.	Emetic Agent (ester) Dose	Emetic events/ No. expts.	Latent period min.	Route of administration	Control expts. Emetic events/ No. expts.	Control expts. Latent period min.
Lidocaine							
0.4 % 30 ml, stomach	56	isobutyl.-valine, 2 ml	0/1	–	stomach	1/1	5
0.5 % 10 ml, stomach	56	isopropyl. phenylal., 2 ml	0/1	–	stomach	3/3	5–10
		isopropyl.-phenylal., 5 ml	1/1	10	stomach		
0.5 % 20 ml, duod	39	diisoprop.-glutamic acid, 5 ml	1/1	40	duodenum	1/1	4
0.5 % 20 ml, stomach	56	diisoprop.-glutamic acid, 2 ml	0/1	–	stomach	1/1	4
		isopropyl.-phenylal., 2 ml	0/1	–	stomach		
2.0 % 10 ml, stomach	56	isopropyl.-phenylal., 1 ml	0/1	–	stomach	1/2	
Atropine,							
0.5 mg, 10 ml, duod	89	isobutyl.-valine, 2 ml	0/1	–	duodenum	9/12	5–12
1 0 mg, 10 ml, stomach	88	diisoprop.-glutamic cid, 2 ml	0/3	–	stomach	1/1	4
		isopropyl.-phenylal., 4 ml	0/2	–	stomach	3/4	8–12
1.0 mg, 10 ml, duod.	89	isopropyl.-phenylal., 2 ml	0/2	–	duodenum	3/3	2–8
	95	diisoprop.-glutamic acid, 4 ml	2/2	5–6	duodenum	2/2	4–6
1.0 mg, 20 ml, duod.	89	diisoprop.-glutamic acid, 2 ml	0/2	–	duodenum	4/4	4–10
2 0 mg, 10 ml, duod.	95	isopropyl.-phenylal., 4 ml	1/1	7	duodenum	3/3	5–10
0.5 mg, subc.	88	diisoprop.-glutamic acid, 4 ml	1/1	2	stomach	2/3	4–5
	89	diisoprop.-glutamic acid, 2 ml	2/2	4–50	duodenum		
	95	isobutyl.-valine, 4 ml	0/2	–	duodenum	3/4	4–6
1.0 mg, subc.	88	diisoprop.-glutamic acid, 4 ml	0/2	–	stomach		
	95	diisoprop.-glutamic acid, 4 ml	3/3	3	duodenum		
2 0 mg, subc.	83	diisoprop.-glutamic acid, 2 ml	0/1	–	stomach	1/1	15

interval before emesis ranged from 4 to 6 minutes. Dog 180 showed retching at all dose levels but vomiting only twice with the 5 g dose (2/4 experiments) 8 mm. after administration. Diethyl-ester of glutaric acid was given to dog 180 only in 9 intraduodenal administrations of 5 experiments with doses varying between 0.2 and 7 g. Retching was frequent at all dose levels but vomiting was induced only once by the 7 g dose 8 min. after administration.

Blockade of emetic response

All experiments performed with lidocaine and atropine to block the emetic action of the various esters are shown in table 5 Gastric or duodenal administration of 0 4–2.0 / lidocaine (10–30 ml) caused a marked reduction of frequency of emesis. In 7 experiments on 2 dogs vomiting occurred only twice (29 /) whereas in the control experiments with the same amount of ester vomiting was provoked in 7 of 8 experiments (88 %). However in the 2 experiments where the emetic effect was not blocked, the period for occurrence of emetic response was prolonged considerably In experiments with gastric administration, there was free passage to the duodenum. Only first time administrations of an experiment are included in the controls in order to prevent interference with the results of the refractory period. Administration of 0.5 1 0 and 2.0 mg atropine in 10–20 ml saline locally in the stomach or duodenum inhibited the emetic response to the same extent as the local anaesthetic. Three emetic events in 13 administrations (23 /) on 3 dogs occurred with atropine and 2 or 4 ml of the various esters but without atropine the frequency of emesis was 25 out of 29 administrations (86 /). Subcutaneous administration of 0.5 1 0 and 2.0 mg atropine was much less effective in inhibiting the emetic effect of locally administered esters. Vomiting was initiated in 6/11 administrations (55 /) on 4 dogs with atropine and in 14/17 administrations (82 /) in control experiments on those dogs.

Influence on acidity and gastric acid secretion.

A. *Influence on gastric pH*

The pH of gastric content is raised markedly during gastric administration. In dog 56, the pH was measured in situ during 4 administrations of 1 or 2 ml (3 and 1 experiments respectively) of isopropyl-ester of phenylalanine (table 6) A rapid decrease of acidity was noted with the pH remaining at 7.0–7 6 for 5–15 min. However the effect was rather short lasting and the pH returned to pre-administration levels within 20–45 minutes.No difference in the rise of pH was observed with the two amounts tested. Administration of a local anaesthetic did not have any influence on the rise of the pH.

B *Stimulation of gastric acid secretion.*

Gastric secretory output (volume) was studied in 5 dogs provided with

Table 6

Influence on gastric acidity and emetic effect by isopropyl-ester of phenylalanine (1 ml + 10 ml saline) with and without simultaneous administration of local anaesthetic (dog 56).

Time min.	Stomach Administration	Stomach pH	Pavlov-pouch secretion volume (ml)
0		2.8	0.7
15	Isopropyl-phenylalanine ➤	2.4	0
25	Emesis ➤	7.4	
30		6.4	1.3
33		3.8	
36		1.8	
45		1.2	0
60		1.5	0
75		2.5	0.7
90		2.3	0
105	Lidocaine, 2 % 10 ml →	2.2	0.5
120	Isopropyl-phenylalanine ➤	2.1	1.1
130		6.2	0
135		7.1	
145		2.5	
150		2.4	0.5
160		2.2	
165		2.5	0.8

Pavlov pouches (36, 39 56 71 90) during administration of the various esters in the stomach (dog 56) or the duodenum (dogs 36, 39 71 90) An increase of basal secretory output was observed only once (dog 90 isopropyl-ester of phenylalanine, 2 ml) of 26 applications of the esters (4 %). 2 ml of isobutyl-ester of valine, 2–5 ml of diisopropyl-ester of glutamic acid and 1 5 ml of isopropyl-ester of phenylalanine were used in these experiments.

The total acid output from the fundic pouch was determined during 37 administrations into the duodenum of two dogs with a Pavlov (dog 141) or a Heidenhain pouch (dog 180) A slight increase of free acid output occurred in only 3 of these applications (8 %) and was of short duration (30 min.). 3–7 ml of ethyl-ester of glycine, 2–3 ml of isopropyl-ester of phenylalanine and 0.5–1 0 ml of diethyl-ester of glutamic acid were used.

To investigate the influence of the esters on release of gastrin the isolated, innervated antrum pouch of two dogs (141 179) was perfused for one hour with a 16 % solution of ethyl-ester of glycine or isopropyl-ester of phenylalanine and total acid output of the totally innervated (dog 141) or vagally denervated fundic pouch (dog 179) determined. Stimulation of acid secretory

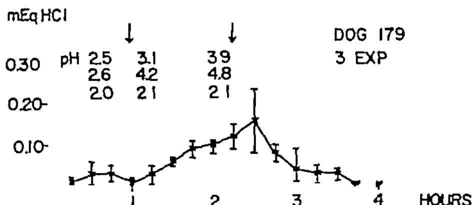


Fig. 1. Stimulation of gastric acid secretion in a Heidenhain pouch dog during perfusion of the isolated antrum pouch with the ethyl-ester of glycine. Perfusion rate = 100 ml/hr Vertical bars indicate range of total acid output. pH before start of perfusion indicates pH of test solution.

output occurred in all 4 experiments with the ester of glycine on dog 141 and in 3 experiments with the ester of glycine and 2 experiments with the ester of phenylalanine on dog 179 (fig. 1). The low pH is explained by use of the hydrochloride of the ester The stimulating effect on gastric acid secretion of the esters during these conditions is much less pronounced than the effect of the corresponding alcohol in that concentration at a similar pH (see ELWIN 1969)

Discussion

Esters of amino acids are amphoteric compounds and have the ability to reduce gastric acidity by binding HCl to the molecule when they enter into the stomach. The ester hydrochloride is then transported into the duodenum where a dissociation of the compound takes place and free HCl molecules will occur again. The esters have a high capacity to combine with HCl molecules exemplified by the fact that 2 ml of isopropyl-ester of phenylalanine can bind about 100 ml of 0.1 N-HCl. An earlier unknown fact became obvious during a study on the capacity of various such esters to reduce gastric acidity – emesis was often initiated as a side effect. Five esters were investigated – ethyl-ester of glycine, diethyl-ester of glutamic acid, diisopropyl ester of glutamic acid, isopropyl-ester of phenylalanine, isobutyl-ester of valine – and these were all found to be potent emetics. The uppermost part

of the duodenum (0–10 cm below the pylorus) was found to be the most sensitive with a declining emetic effect during administrations further down the duodenum (table 4). Below 50 cm from the pyloric sphincter vomiting was never elicited. The stomach proved to be almost insensitive during administrations with occlusion of passage to the duodenum and during perfusion of the isolated, innervated antrum.

Gastric irritants like salicylates (BHARGAVA *et al.* 1963), digitalis (GOLD *et al.* 1950), copper sulphate (WANG & BORISON 1951) and ipecac (WOLF & WOLFF 1946) often cause emesis. The latter authors were able to show that the gastric mucosa is very resistant to the action of local irritants (digitalis) whereas the duodenal mucosa is not. In a later report (WOLF 1949) it was demonstrated that ipecac did not cause retching or vomiting before having reached the duodenum. The emetic action of local irritants is believed to be due to local irritation as such but a more specific mechanism by effect on receptors in the upper gastrointestinal tract cannot yet be excluded. The esters of amino acids may very well act as the local irritants. The appearance of blood-stained diarrhoea after administration far down the duodenum in one dog (95), favours this interpretation. Copper sulphate, a well known local emetic and gastric irritant, was used in some comparative experiments and was found to provoke vomiting from the stomach as well as from the duodenum. Actually in one dog (89) the intraduodenal dose of the emetic agent was less than the oral threshold dose of 40 mg given by WANG & BORISON (1952). Copper sulphate thus seems to have a more pronounced local emetic action than the esters.

Is the emetic action shown by esters of amino acids of local or central origin? According to WANG & BORISON (1952) a central emetic action of an agent on the chemoreceptor trigger zone or the vomiting center cannot be excluded unless ablation of these structures has been undertaken. Diethylester of glutamic acid was the only compound investigated, that could be administered intravenously. The local, duodenal emetic dose of that agent was 0.2 ml in one dog (180) whereas increasing the intravenous dose to 1.2 ml (× 6) did not produce any sign of retching or emesis. This strongly supports the interpretation of a local emetic action in the duodenum of this group of agents but does not exclude the possibility of an additional central emetic action. A local, emetic action is also favoured by the finding that the effect is blocked by local anaesthesia (table 6) and by atropine locally whereas parenteral atropine has much less blocking effect.

Only small differences of the threshold dose of the various esters causing emesis were observed. Diethyl-ester of glutamic acid was the only ester having a considerably lower threshold dose – 0.2–0.3 ml – against 2–4 ml for the other esters.

There was no obvious difference of the emetic effect of the esters and of

the corresponding hydrochlorides. This does not mean, however that the latter compound may be the causative agent as a rapid dissociation of the molecule also occurs after application of the hydrochloride directly in the duodenum.

A local emetic action is apparently not unique for esters of amino acids since esters of some corresponding carboxylic acids did show the same pattern.

In situ measurements of stomach pH revealed that the esters cause a rapid decrease of gastric acidity and could be regarded as potent antacids, but the emetic side effect prevents their use for practical purposes. Besides this the neutralizing effect did not last more than 20–45 minutes.

Gastric acid secretion was not stimulated during stomach or duodenal administration of the esters in amounts causing emesis. On the other hand, perfusion of the isolated, innervated antrum pouch in two dogs clearly stimulated acid output from the innervated or denervated fundic pouch. The latter finding may indicate an action through release of the antral stimulatory hormone, gastrin. To mimic physiological conditions these perfusions were performed at a rather low pH. In other series of experiments it has been shown that some alcohols (WOODWARD *et al* 1957 ELWIN 1969) as well as some amino acids (ELWIN & UVNÄS 1966) stimulate gastric acid secretion through activation of the antral gastrin mechanism. According to the present findings the effect on the antral mechanism of esters of amino acids is much less pronounced than the effect of a corresponding stimulating alcohol and about equal to the effect of a corresponding stimulating amino acid at the pH used.

Acknowledgement

This research was supported by grants from the Swedish Medical Research Council (No. B 67 14x-98-03) and from Karolinska Institutet.

REFERENCES

Andersson, S. & C.-E. Elwin: The emetic action of esters of some amino acids. *Acta physiol. scand.* 1957 42, Suppl. 145.

Bhargava, K. P O Chandra & D. R. Verma: The mechanism of the emetic action of sodium salicylate. *Brit J Pharmacol.* 1963, 21 45–50.

Borison, H. L. & S. C. Wang: Physiology and pharmacology of vomiting. *Pharmacol. Rev* 1953, 5, 193–230.

Elwin, C.-E. & B. Uvnäs: Distribution and local release of gastrin. *UCLA Forum Med. Sci.* 1966, 5 69–82.

Elwin, C. E.. Stimulation of gastric acid secretion by irrigation of the antrum with some aliphatic alcohols. *Acta physiol. scand.* 1969 75 1–11

Gold, H McK. Cattell, W Modell, T Greiner & R. Guevara. Local emetic activity of glycosides of the digitalis series. *J Pharmacol.* 1950, 98, 337–345

Uvnäs, B., S. Andersson, C. E. Elwin & A. Malm: The influence of exclusion of the antrum-duodenum passage on HCl secretion in Pavlov pouch dogs. *Gastroenterology* 1956, 30 790–804.

Wang, S. C. & H L. Borison Copper sulphate emesis study of afferent pathways from gastrointestinal tract. *Am. J Physiol.* 1951, 164 520–526.

Wang, S. C. & H. L. Borison. A new concept of organization of the central emetic mechanism. recent studies on the sites of action of apomorphine, copper sulfate and cardiac glycosides. *Gast oenterology* 1952, 22, 1–12.

Wolf, S. & H. G Wolff Action of drugs and various chemical agents on the gastric mucosa and gastric function in man. *N Y State J Med.* 1946, 46, 2509–2512.

Wolf S. Studies on nausea, effects of ipecac and other emetics on human stomach and duodenum. *Gastroenterology* 1949 12, 212–218.

Woodward, E. R., C. Robertson, H. R. Ruttenberg & H. Schapiro: Alcohol as a gastric secretory stimulant. *Gastroenterology* 1957 32, 727–737

Acta pharmacol. et toxicol. 1970, 28 177–183

From the Industrial Toxicology Research Centre, Lucknow India

Experimental Production of Early Brain Lesions in Rats by Parenteral Administration of Manganese Chloride

By

Satya V Chandra and S. P Srivastava

(Received August 18, 1969)

Abstract: Attempts have been made to produce early brain lesions in rats by the daily intraperitoneal administration of manganese chloride (8 mg/kg) for a period of 180 days. Neuropathological examination performed at various intervals showed scattered neuronal degeneration in the cerebral and cerebellar cortex at 120 days which increased in intensity up to 180 days. The manganese concentration in the brain also showed significant increase. The extent of the brain lesions was directly related to the amount of manganese in the brain tissue which increased with time.

Key-words: Brain – brain injury acute – manganese.

Excessive manganese in the human body is known to cause degeneration of the basal ganglia of the brain (CANAVAN *et al.* 1934) It has also been established that manganese enters the body mainly via the pulmonary route and produces lesions in the extrapyramidal system, the base of the brain and the upper part of the spinal cord (WHITLOCK *et al.* 1966) The development of brain lesions and the mechanism responsible for their production is not understood. This experiment was undertaken in the rat in order to study early brain lesions produced by manganese and the relation between the manganese concentration and the development and severity of the pathological lesions.

Materials and Methods

Ninety male albino rats of I. T R. C. colony (average weight 150) were reared under normal laboratory conditions and fed a stock diet which contained only small traces of manganese. The animals were divided into two groups. Group I consisted of 30 control animals into which 0.5 ml distilled water was injected and group II consisted of sixty animals into which manganese chloride in aqueous solution (8 mg/kg) was injected intraperitoneally daily for 180 days. Six animals from group I and 24 animals from group II died during the course of the experiment; the cause of their death however

could not be ascertained. Four animals from group I and six from group II were sacrificed at 30 days intervals up to 180 days and during this period the animals did not develop any unusual neurological symptoms. Animals that died during the experiment and which were sacrificed at various intervals were autopsied and the brains removed and examined for gross pathological changes. Th. brains from half the animals from each group were kept in neutral buffered formalin for histopathological studies while in the remainder of the specimens estimation of the manganese content was done by the method of SRIVASTAVA et al. (1969). For histopathological studies, blocks of tissue were embedded in paraffin and sections were cut at 10 μ. The sections were stained with hematoxylin and eosin, carbol thionine for Nissl substance, Holzer's stain for glia fibres, Bielschowsky's stain for neurofibrils and Weil's stain for myelin sheath (McMANUS & MOWRY 1960)

Estimations of the manganese content were done in brain tissue of experimental and control animals at 60 120 and 180 days as well as in animals which died during the experiment. The results of manganese content in the animals dying up to 60 days are pooled together with the results of animals sacrificed at 60 days and similarly the results of dead animals up to 120 and 180 days have been pooled with the results of animals sacrificed at 120 and 180 days respectively

To determine the number of degenerated neurons in the brain cortex, nerve cell counts were performed by the following method.

A paper diaphragm with a circular cut about 4 mm in diameter in the centre was inserted in a number 10 eye piece. Sections were put under magnification × 40, and 1000 degenerated and normal neurons were counted from one to the other side of the cortex, in the same way as in differential white cell counts. The average numbers of degenerated neurons were calculated from each group. Counts were performed at different periods of the experiment in both groups.

Results

Pathological findings

Macroscopic. The meninges were smooth and shining basal vessels and cranial nerves were normal in all the animals in groups I and II. No gross pathological change was observed in the brain and spinal cord of group I animals In group II no gross pathological changes were seen in the brain and spinal cord up to 120 days at 150 and 180 days, the brain appeared paler than normal.

Microscopic Sections of the brain from group I animals did not reveal any pathological change (fig. 1) In group II (manganese chloride injection) sections of brain did not reveal any pathological change up to 90 days. At 120 days scattered neuronal degeneration in the cerebellar and cerebral cortex was seen. Nissl granules were absent in these cells. A few oligodendroglia appeared to be swollen. The blood vessels did not show any abnormality except a mild congestion in one rat. The ventricular ependyma and choroid plexus were normal in appearance while the white matter was found to be normal and the myelin stain revealed normal structure. Sections from the brain at 150 days showed vacuolated nerve cells in the cerebral and



Fig. 1. Photomicrograph of cerebral cortex of a control rat, showing normal structure of brain tissue. Hematoxylin and eosin. Magnification × 400.

cerebellar cortex, and degenerative changes in the form of chromatolysis, the nuclei were shrunken and in some cases pyknotic. No myelin sheath degeneration was observed. There was swelling of the oligodendroglia. The meninges, ventricular ependyma, choroid plexus and blood vessels did not reveal any abnormality Sections from the brain at 180 days showed a fairly large number of degenerated neurons scattered in the cortex. In some of these cells Nissl granules were absent and the cytoplasm was pale and vacuolated, the nucleus in degenerated neurons was shrunken and pyknotic and in some cases had completely disappeared. There was marked swelling of the oligodendroglia (fig. 2) The basal ganglia did not reveal any pathological change. No myelin sheath degeneration was noted. The meninges, ventricular ependyma, choroid plexus and blood vessels appeared to be normal.

The nerve cell count at various intervals showed that the percentage of degenerated neurons in the animals of group I was 5 4 and in group II this was almost the same as in group I up to 90 days thereafter it increased to 17.3 at 120 days, 31 9 at 150 days and 43 3 at 180 days (table 1) This indicates that degenerative lesions in the brain due to manganese are present at 120 days and the severity increases with time thus confirming the microscopic findings of maximum lesions in the brain at 180 days.



Fig. 4. Photomicrograph of cerebral cortex of rat after intraperitoneal injections of manganese chloride (8 mg/kg body weight) for 180 days, showing degenerated neurons and swollen oligodendroglia. Hematoxylin and eosin. Magnification × 400.

Manganese concentration in brain (mg/g dry weight of brain tissue)

The results on the manganese content in the brains of both the groups are shown in table 2. The average manganese content in brains of animals of

Table 1

Average number of degenerated neurons in the cortex of brain in control and manganese injected rats.

Neurons	Control	Manganese injected rats					
		Days					
	0	30	60	90	120	150	180
Degenerated	54	54	53	50	173	319	433
Normal	946	946	947	950	827	681	567
	(10)	(4)	(4)	(4)	(4)	(4)	(4)
Percent	5.4	5.4	5.3	5.0	17.3	31.9	43.3

Figures in brackets indicate number of animals.

Table 2

Manganese content in brain of control and manganese injected rats.

	Control	Manganese injected rats		
Manganese mg/g dry weight of brain tissue	0	60 days	120 days	180 days
Mean value ± S. E. M ...	2.253 ± 0.2190 (7)	3 784 ± 0.2445 (6)	4.863 ± 0.1378 (6)	6.849 ± 0.1502 (6)
Statistical significance of the difference –				
1. from control	–	Significant $P < 0.05$	Highly significant $P < 0.01$	Highly significant $P < 0.01$
2. from 60 days	–	–	Not significant	Highly significant $P < 0.01$
3. from 120 days	–		–	Significant $P < 0.05$

Figures in brackets indicate number of animals.

group I was 2.2530 in the animals of group II up to 60 days this was 3 7844 up to 120 days 4 8636 and up to 180 days 6.849 Statistical analysis showed that the difference in manganese content between the control and the ex perimental group up to 120 and 180 days is highly significant while it is just significant at 60 days.

On comparing the number of degenerated neurons and the amount of manganese content it appears that the severity of lesions is directly related to the manganese concentration in the brain. At 120 days when microscopic lesions are seen in the brain, the amount of manganese is 4 86 which is highly significant as compared to group I. At 180 days maximum lesions are observed in the brain i. e. when the amount of manganese has increased to 6.84 mg/g dry weight of brain tissue.

Discussion

The early histological changes in the human brain due to manganese toxicity are not known. The reports available are only of advanced cases where the condition resembles Parkinsonism (CANAVAN *et al.* 1934

PARNITZKE & PFIFFER 1954) KIRCHER *et al.* (1960) studied the electroencephalographic pattern in patients with manganese poisoning and observed that the lesions were more numerous in the frontal lobes than in the basal ganglia or the cerebellum. Further it has also been observed that in manganese toxicity (RODIER 1955) there is a period of mental excitement in human subjects before the actual manifestations of lesions in the extrapyramidal system How far these changes are related to early lesions is not known. In the present experiment it has been possible to produce lesions in the cerebral and the cerebellar cortex due to manganese toxicity in which the extra pyramidal system seems to escape injury

The lesions of the basal ganglia in experimental animals produced by MELLA (1924) and PENTSCHEW *et al.* (1963) and the cortical lesions produced by SAXENA (1967) in our laboratory did not demonstrate any relationship between the amount of manganese present and the severity of lesons in the brain. The present experiments however have demonstrated that the maximum number of degenerated neurons are present when the amount of manganese in the brain is at a maximum, thus indicating that the extent of damage to the brain cells is directly related to the amount of manganese present in the brain. The period up to 120 days appears to be a threshold for the appearance of microscopic lesions. It is possible that very early pathological changes may be present which can not be detected by light microscopy or that initially manganese produces biochemical disturbances rather than visible structural changes.

The present experiment supports the hypothesis that in experimental animals damage to the brain cells can be caused directly by soluble manganese. It remains to be determined whether manganese has a direct toxic effect on the brain tissue or whether excess manganese in the blood conjugates with protein molecules to form a toxic compound which is responsible for causing damage to brain cells. This requires further investigation. In a case of hepatolenticular degeneration, however an abnormal protein in the liver was de monstrated which had an abnormally high copper binding property (CUMINGS 1959) The possibility of a vascular pathogenesis (STADLER 1936) is also ruled out as the blood vessels in our experiment did not show any abnor mality

Acknowledgement

Thanks are due to Dr S. H. Zaidi, Director Industrial Toxicology Re search Centre, for his valuable suggestions and guidance throughout the course of the experiments. The technical assistance of Shri R. S. Srivastava and Shri Mulkraj is gratefully acknowledged. We would like to thank Mr George for the statistical analysis and Mr Musleh Ahmad for the microphotography

REFERENCES

Canavan, M. M. S. Cobb & C. K. Drinker Chronic manganese poisoning. *Arch. Neurol. Psychiat.* 1934 32, 501–512.

Cumings, John N. *Heavy metals and the brain.* Blackwell scientific publications. Oxford 1959

Kircher M. M. J. Ritter Fecad Marti & Belaomane: Frequency of cortical lesions in manganese poisoning. *Arch. Malad. Professionelles* Paris 1960. Quoted from *Bull. Hyg.* 1961 36, 994

McManus, J. F. A. & R. W. Mowry *Staining methods Histological and histochemical.* Paul B. Hoeber Medical division of Harper & Brothers, New York, 1960.

Mella, H. *Arch. Neurol. Psychiat.* (Chicago) 1924 11, 405 Quoted by Donald Owen. Manganese poisoning, *Lancet* 1934, 2, 989–990.

Parnitzke, K. H. & J. Peiffer Zur Klinik and pathologischen Anatomie chronischer Braunsteinvergiftung. *Arch. Psychiat. Nervenkr.* 1954, 192, 405 Quoted from *J. Neuropath. exp. Neurol.* 1963 22, 488–499

Pentschew A., F. F. Ebner & R. M. Kovatch. Experimental manganese encephalopathy in monkeys. *J. Neuropath. exp. Neurol.* 1963, 22, 488–499

Rodier J.: Manganese poisoning in Moroccan mines. *Brit. J. Industr. Med.* 1955, 12, 21–31.

Saxena, K. The acute effect of manganese chloride on the central nervous system of Rats. *Ind. J. Industr. Med.* 1967 13 66–72.

Srivastava, S. P. K. P. Pandya & S. H. Zaidi. Determination of the manganese in blood and biological materials. *Analyst.* 1969 94, 823–827

Stadler H. Z., *Ges. Neurol. Psychiat.*, 1963, 154 62. Quoted from Greenfield's: *Neuropathology* Edward Arnold Ltd., London, 1965, p. 271.

Whitlock, C. M., S. J. Amuso & J. B. Bittenbender Chronic neurological disease in two manganese steel workers. *Am. Indust. Hyg. Ass. Jour.* 1966, 27 454–459

Acta pharmacol. et toxicol. 1970 28, 184–191

From the Department of Pharmacology Royal Danish School of Pharmacy
Copenhagen, Denmark (Professor Erik Jacobsen, M. D.)

The Blood Pressure Response Following Angiotensin in Nephrectomized, Urethanized Rats

By

Annette Skinhøj

(Received October 21, 1969)

Abstract. The increase in blood pressure is short lasting in normal rats after supramaximal repeated doses of angiotensin and long lasting in nephrectomized rats anaesthetized with urethane. Twenty minutes after the administration of 2.5-7.5 μg per kg hypertensin® intravenously during 7.3 min. the increase in blood pressure in 4 animals nephrectomized 4-7 hours previously was 7 ± 1 mmHg and in 6 animals nephrectomized 18-24 hours previously was 13 ± 2 mmHg. No prolonged effect on the blood pressure was seen in animals nephrectomized 30-45 min. previously or in nephrectomized animals anaesthetized with amobarbital. Epinephrectomy or 1 mg per kg phenoxybenzamine prevented the long lasting effect of angiotensin. No correlation was found between the initial blood pressure and the production of a prolonged rise in blood pressure.

Key-words. Angiotensin – blood pressure – nephrectomy – urethane.

Many investigators have observed different blood pressure curves following renin injections into normal and nephrectomized animals. In the latter a higher peak pressure and a more prolonged duration of the rise in blood pressure is seen (Tigerstedt & Bergman 1898) This effect is found in different species and seen both in conscious and anaesthetized animals.

In contrast to the general agreement about the effect of renin injections, there is some disagreement in the literature about the effect of nephrectomy on changes in blood pressure after angiotensin injections. Some investigators have found an increased peak pressure following a single injection of angiotensin into nephrectomized animals (Page & Helmer 1940· McCubbin & Page 1954 Gross & Sulser 1956) On the other hand Blaquier *et al.* (1962) and Bing & Magill (1963) failed to find such an increase.

In addition to the effect on the peak pressure Gabelman & Rondell (1966) described a prolonged rise in blood pressure following angiotensin injections

in nephrectomized rats anaesthetized with urethane. The prolongation was absent in sham operated animals but still found after ganglionic blockade. The effect was shown not to be due to a delayed elimination of angiotensin from the circulating blood.

The purpose of the present work was to investigate the mechanism of this long lasting effect of angiotensin.

Methods

White male and female rats weighing 170-250 g from different commercial sources were used at random. The rats were anaesthetized with urethane 1.75 g per kg subcutaneously or with an initial dose of 125 mg per kg amobarbital (pentymalum NFN) sodium intraperitoneally followed by 12.5 mg per kg intravenously if necessary. Intravenous injections were administered through the external jugular vein, and blood pressure recordings were obtained from the common carotid artery by means of a mercury manometer

Bilateral nephrectomy or bilateral adrenalectomy was performed through lumbar incision under ether anaesthesia 18-24 hours before the experiments unless specially noted. The animals were allowed free access to food and water In experiments started less than 45 min. after the nephrectomy the animals were operated upon under urethane or amobarbital anaesthesia.

Ganglionic blockade. Pentolinium (pentolonium NFN) bitartrate 10 mg per kg (ansolysen®) was injected intramuscularly 30-45 min. before the experiment.

Adrenergic-α-blockade Phenoxybenzamine (benzylytum NFN) 1 mg per kg (blocadren®) was injected slowly intravenously and the injection repeated after 10 min. If the effect on the blood pressure of intravenous injection of 50 ng noradrenaline was not almost abolished 60 min. after the second dose, a third dose was given.

Angiotensin injections. Hypertensin® (angiotensinamidum NFN) dissolved in 0 9 % NaCl solution in concentrations ranging from 1 µg per ml to 5 µg per ml was injected with a 0.5 ml tuberculin syringe as follows: 10 × 10 µl injected at 10 sec. intervals plus 10 × 10 µl injected at 15 sec. intervals plus 10 × 10 µl injected at 20 sec. intervals, the total period of injections thus lasting 440 sec.

Noradrenaline injections. Noradrenaline bitartrate dissolved in 0.9 % NaCl solution containing 10 µg noradrenaline base per ml was injected as follows: 10 × 10 µl at 5 sec. intervals plus 30 × 10 µl injected at 10 sec. intervals, the total period of injections lasting 350 sec.

Results

Unoperated rats injected with angiotensin in a total amount of 0 75 to 1.5 µg during 7.3 min. showed a rapid rise in blood pressure followed by a slow decrease as the injection rate was diminished. Less than 5 min. after the last injection the blood pressure had returned to the pre-injection level

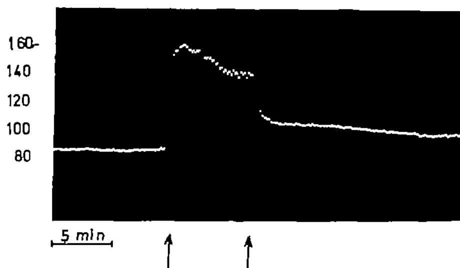


Fig. 1. Typical blood pressure curve showing prolongation of the blood pressure rise after angiotensin administration. *The rat is nephrectomized 18 hours before the ex* periment and anaesthetized with urethane. The arrows indicate the first and the last injection of angiotensin in a concentration of 5 µg per ml.

Table I

Blood pressure effects of angiotensin in urethane anaesthetized rats.

Treatment	Number of animals	Initial B. P mmHg, mean ± S. E. M.	Max. B. P increase mmHg, mean ± S. E. M.	B. P increase 70 min. after last angiotensin inj. mmHg, mean ± S. E. M.
Untreated controls	3	100 ± 9	37 ± 6	0 ± 0
Controls, ganglionic blockade	2	38* 56	52 52	−6 4
30–45 min. nephrect.	4	78 ± 14	49 ± 9	−4 ± 2
4–7 hrs nephrect.	3	101 ± 8	55 ± 4	7 ± 1
18–24 hrs nephrect.	6	92 ± 11	59 ± 6	13 ± 2
18–24 hrs nephrect. + ganglionic blockade	2	56 64*	86 76	16 16
18–24 hrs nephrect. + adr.-α-blockade	5	48 ± 5	40 ± 4	0 ± 0
18–24 hrs nephrect. + adrenalect.	5	63 ± 5	68 ± 5	0 ± 0

If only two animals are used the data for each singl animal are given.

Table 2

Blood pressure effects of angiotensin in amobarbital anaesthetized rats.

Treatment	Number of animals	Initial B. P mmHg, mean ± S. E. M.	Max. B. P increase mmHg, mean ± S. E. M.	B. P increase 20 min after last angiotensin inj mmHg, mean ± S. E. M.
Untreated controls	2	104 130*	60* 44	−2* −2*
18–24 hrs nephrect.	5	98 ± 5	51 ± 6	−2 ± 2

If only two animals are used the data for each animal are given.

(table 1) Supramaximal doses were used, and the peak pressure during the injections was independent of the injected dose. Ganglionic blockade did not alter the time course of the blood pressure curve, and no difference was observed between animals anaesthetized with amobarbital or urethane (table 1 & 2).

Rats bilaterally nephrectomized 30–45 min. before the administration of angiotensin showed the same pattern as normal rats.

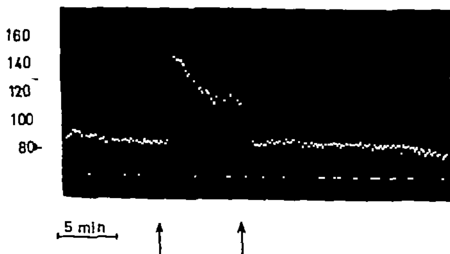


Fig. 2. Typical blood pressure curve showing no prolongation of the blood pressure increase after angiotensin administration. In this experiment rat anaesthetized with urethane and nephrectomized and adrenalectomized, 18 hours before the experiment was used. The arrows indicate the first and the last injection of angiotensin in a concentration of 5 μg per ml.

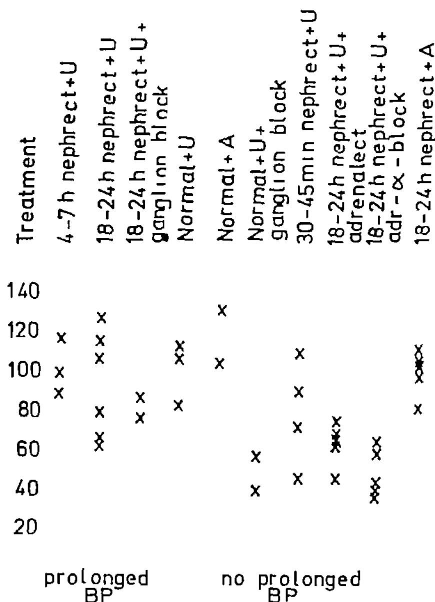


Fig. 3. The figure shows the blood pressure of each experimental animal before the angiotensin injections. The left column includes animals showing increased blood pressure 20 min. after the last angiotensin injection. The right column includes the animals with no prolongation of the blood pressure rise.

U- urethane anaesthetized animals.

A. amobarbital anaesthetized animals.

Animals injected with angiotensin 4–7 hours after the nephrectomy and anaesthetized with urethane showed a small increase in the blood pressure level after the angiotensin injections had been discontinued, and in rats nephrectomized 18–24 hours previously and anaesthetized with urethane, the blood pressure remained distinctly increased throughout the observation period i. e. 20 min. after the last injection (fig. 1)

The prolongation was independent of the time of exposure to urethane which varied between 1 and 4 hours. If the total amount of angiotensin injected was less than 0.3 µg no significant prolongation was seen, but after larger doses no correlation between the injected dose and the blood pressure level 20 min. after discontinuation of the injections could be observed

Treatment with ganglion blocking agents did not prevent the prolongation.

If the adrenals were removed with the kidneys the prolonged blood pressure increase was completely prevented (fig. 2) The same effect was obtained with adrenergic-α-blocking agents (table 1)

When noradrenaline was injected in doses giving the same peak pressure as the angiotensin injections, no prolonged effect in blood pressure was seen in nephrectomized rats.

Rats nephrectomized 18–24 hours previously and anaesthetized with amobarbital failed to show any persistent blood pressure increase when injected with angiotensin in the same dosage as used above (table 2)

No correlation could be seen between the initial blood pressure and the presence of a prolonged increase in blood pressure after angiotensin injections (fig. 3)

Discussion

The prolonged increase in blood pressure of nephrectomized rats after injections of angiotensin, mentioned by GABELMAN & RONDELL (1966) is also demonstrated here. The effect was shown by GABELMAN & RONDELL to persist when angiotensin could no longer be detected in the blood, so that the difference between normal and nephrectomized animals could not be due to a delayed disappearence of angiotensin from the circulation.

In the present work the prolongation of the blood pressure increase is shown to depend on the presence of the adrenals and of active adrenaline α-receptor sites. The present results from the experiments with animals treated with ganglion blocking agents are similar to those of GABELMAN & RONDELL, and show that the prolonged effect of angiotensin is independent of intact peripheral ganglionic transmission.

The possibility that the deprivation of endogenous angiotensin in the nephrectomized state would favour a binding of the injected angiotensin to

the receptor sites and thereby protect it from destruction cannot be ignored, but a direct vascular action of angiotensin is unlikely since the prolonged rise in blood pressure is abolished by adrenergic α blocking agents, suggesting that the catecholamines are involved. An action via the catecholamines released from the adrenal medulla is quite likely as animals both nephrectomized and adrenalectomized do not show any prolongation. Angiotensin is known to cause an output of catecholamines especially adrenaline from the adrenals in animals. In rats CESSION & CESSION-FOSSION (1963), using perfusion of isolated adrenals, found an increased catecholamine level in the outflow after addition of angiotensin to the perfusion fluid. *In vivo* the effect on the adrenal medulla has been shown in cats (FELDBERG & LEWIS 1964 LEWIS & REIT 1966 STASZEWSKA BARCYAK & VANE 1967) in dogs (STASZEWSKA BARCYAK & VANE 1967 PEACH *et al.* 1966), and in guinea pigs (PIPER & VANE 1967) In cats and dogs the increased blood level of catecholamines could only be seen during the first 5–20 min. of a continuous infusion of angiotensin (STASZEWSKA BARCYAK & VANE 1967 PEACH *et al.* 1966) and a similar release was found after a renin injection into a nephrectomized cat by FELDBERG & LEWIS (1965). As the catecholamine release was still found after hexamethonium treatment, FELDBERG & LEWIS (1965) ascribed the effect to a direct action of angiotensin, not involving acetylcholine. In the present work the prolonged rise in blood pressure after angiotensin injections is seen only in urethane anaesthetized rats. It could be expected that the effect would also be present in the animals anaesthetized with amobarbital, as a barbiturate (pentobarbital) (mebumalum NFN) was used as anaesthetic in some of the experiments cited above. On the other hand, a quantitative comparison between the release after the different anaesthetics has not been performed.

SPRIGGS (1965) showed in rats that urethane affects the catecholamine stores. During the first three hours of urethane anaesthesia, but not after pentobarbital or phenobarbital (phenemalum NFN), he found a decrease in the content of catecholamines in the adrenals and an increase in the heart.

If the prolonged angiotensin induced blood pressure rise in nephrectomized rats is caused by catecholamines from the adrenals, it must be due to an altered release since no prolongation of the blood pressure rise has been seen after injections of adrenaline or noradrenaline (GABELMAN & RONDELL 1966 and the present work) into nephrectomized rats. Hence the disappearence rate of catecholamines from the circulating blood is not altered by nephrectomy

Acknowledgement

I would like to thank Alfred Benzon A/S for kindly supplying blocadren®

REFERENCES

Bing, J & F Magill. Potentiation of sensitivity to renin in normal rats by cross circulation with nephrectomized. *Acta path. microbiol scand.* 1963 59 485–490.

Blaquier P S. W Hoobler J Schroeder A. Gomez & T Kreulen. Effect of bilateral nephrectomy on pressor response to renin. *Amer J Physiol.* 1962, 203 339–343.

Cession, G. & A. Cession-Fossion. *Action cortico-surrénalienne de l'angiotensine chez le rat. C. R. Soc. Biol.* 1963 157 1830–1832.

Feldberg, W & G. P Lewis: The action of peptides on the adrenal medulla. Release of adrenaline by bradykinin and angiotensin. *J Physiol.* (Lond.) 1964 171, 98–108.

Feldberg, W & G. P Lewis: Further studies on the effects of peptides on the supra renal medulla of cats. *J Physiol.* (Lond.) 1965, 178, 239–251.

Gabelman, E. H. & P A. Rondell. Protracted pressor response to angiotensin after bilateral nephrectomy in rats. *Circul Res.* 1966, 18 705–713.

Gross, F & F Sulser Wirkungsverstärkung von Renin und Nierenextrakt an der nierenlosen Ratte. *Naunyn-Schmiedebergs Arch. exp Path. Pharmacol.* 1956, 229 338–347

Lewis, G. P & E. Reit: Further studies on the actions of peptides on the superior cervical ganglion and the suprarenal medulla. *Brit. J Pharmacol.* 1966, 26 444–460.

McCubbin, J W & I. H. Page: Renal inhibition of pressor responses to drugs. *Circul Res.* 1954 2, 35–40.

Page, I. H. & O. M. Helmer Angiotonin-activator renin and angiotonin inhibitor and the mechanism of angiotonin tachyphylaxis in normal, hypertensive and nephrectomized animals. *J exp. Med.* 1940, 71, 495–519

Peach, M. J W H. Cline & D T Watts. Release of adrenal catecholamines by angiotensin 2. *Circul. Res.* 1966, 19 571 575.

Piper P J & J R. Vane: The assay of catecholamines released into the circulation of the guinea-pig by angiotensin. *J Physiol.* (Lond.) 1967 188 20–21.

Spriggs, T L. B The effects of anaesthesia induced by urethane or phenobarbitone upon distribution of peripheral catecholamines in the rat. *Brit. J Pharmacol.* 1965 24 752–758.

Staszewska-Barczak, J & J R. Vane: The release of catecholamines from the adrenal medulla by peptides. *Brit J Pharmacol.* 1967 30 655–667

Tigerstedt, R. & P G Bergman. Niere und Kreislauf *Skand Arch Physiol.* 1898, 8 223–271.

the receptor sites and thereby protect it from destruction cannot be ignored, but a direct vascular action of angiotensin is unlikely since the prolonged rise in blood pressure is abolished by adrenergic α-blocking agents, suggesting that the catecholamines are involved. An action via the catecholamines released from the adrenal medulla is quite likely as animals both nephrectomized and adrenalectomized do not show any prolongation. Angiotensin is known to cause an output of catecholamines especially adrenaline from the adrenals in animals In rats CESSION & CESSION-FOSSION (1963) using perfusion of isolated adrenals, found an increased catecholamine level in the outflow after addition of angiotensin to the perfusion fluid. *In vivo* the effect on the adrenal medulla has been shown in cats (FELDBERG & LEWIS 1964 LEWIS & REIT 1966 STASZEWSKA BARCYAK & VANE 1967) in dogs (STASZEWSKA-BARCYAK & VANE 1967 PEACH *et al.* 1966) and in guinea pigs (PIPER & VANE 1967) In cats and dogs the increased blood level of catecholamines could only be seen during the first 5–20 min. of a continuous infusion of angiotensin (STASZEWSKA-BARCYAK & VANE 1967 PEACH *et al.* 1966) and a similar release was found after a renin injection into a nephrectomized cat by FELDBERG & LEWIS (1965) As the catecholamine release was still found after hexamethonium treatment, FELDBERG & LEWIS (1965) ascribed the effect to a direct action of angiotensin, not involving acetylcholine. In the present work the prolonged rise in blood pressure after angiotensin injections is seen only in urethane anaesthetized rats. It could be expected that the effect would also be present in the animals anaesthetized with amobarbital, as a barbiturate (pentobarbital) (mebumalum NFN) was used as anaesthetic in some of the experiments cited above. On the other hand, a quantitative comparison between the release after the different anaesthetics has not been performed.

SPRIGGS (1965) showed in rats that urethane affects the catecholamine stores. During the first three hours of urethane anaesthesia, but not after pentobarbital or phenobarbital (phenemalum NFN) he found a decrease in the content of catecholamines in the adrenals and an increase in the heart.

If the prolonged angiotensin induced blood pressure rise in nephrectomized rats is caused by catecholamines from the adrenals, it must be due to an altered release since no prolongation of the blood pressure rise has been seen after injections of adrenaline or noradrenaline (GABELMAN & RONDELL 1966 and the present work) into nephrectomized rats. Hence the disappearence rate of catecholamines from the circulating blood is not altered by nephrectomy

Acknowledgement

I would like to thank Alfred Benzon A/S for kindly supplying blocadren®

tubular mechanisms in the mouse. The significant uptake of decamethonium in the liver and kidney of mice also raised the question whether these organs would take up decamethonium by qualitatively identical processes.

The object of the present study* was to examine more closely the *in vitro* uptake of ^{14}C-decamethonium in the mouse kidney with particular reference to the influence of the duration of incubation and the external decamethonium concentration on the uptake. Experiments were carried out under various metabolic conditions to decide whether the uptake required energy. It was further investigated, whether the uptake was influenced by the presence of other bis- and monoquaternary ammonium compounds considered as potential inhibitors. The possibility of metabolic transformation of ^{14}C-decamethonium was investigated by the method of paper chromatography (BRØEN CHRISTENSEN & HOLM 1969). For the sake of comparison with ^{14}C-decamethonium, the renal uptake of ^{14}C-hexamethonium was also studied.

Methods

Materials.

The following radiochemicals were used. ^{14}C-methyl-decamethonium dibromide (specific activity 20.9 mci/mM) and ^{14}C-sucrose (specific activity 30.9 μci/mg) were obtained from the Radiochemical Centre, Amersham, England. ^{14}C-methyl-hexamethonium dichloride (specific activity 1.55 mci/mM) was provided by the New England Nuclear Corp., Boston, U. S. A.

The following unlabelled compounds were used: decamethonium dibromide (syncurine ®) and decamethonium diiodide were obtained from Burroughs Wellcome & Co. Hexamethonium dibromide was received from May & Baker Ltd. Tetraethylammonium bromide was supplied by Bie & Berntsen, Denmark and N[1]-methylnicotinamide by Sigma, St. Louis, U. S. A. In addition 2.4 (α)-dinitrophenol and iodoacetic acid (both from E. Merck AG Germany) as well as ouabain (Injectabile g-strophanthini, Ph. Nord. 1963) were used.

Preparation of slices.

Two hundred and sixty-two male albino mice (NMRI), ranging in weight from 28–32 g were decapitated and bled. The kidneys were quickly removed, decapsulated and placed on filter paper held firmly between two pieces of frosted glass. Two or three slices from each of the anterior and posterior surfaces were cut free hand with a dry razor blade. This technique should provide a rather constant relation between medullary and cortical tissue. Experiments were carried out to measure the thickness of freshly prepared slices placed on filter paper. The combined slice and paper was trimmed to square shape and weighed. The specific gravity of the kidney tissue was considered to be unity and after subtraction of the weight of the filter paper the

* Part of this study was presented at the XIII Scandinavian Congress Göteborg 1969.

thickness could be calculated from the area and the volume. The calculated thickness of 6 slices ranged from 0.36–0.58 mm (mean: 0.48), but the marked tendency of the tissue to retract indicates that these values are probably too high.

Incubation procedur

Slices from the same animal, generally 10–12 weighing about 200 mg, were placed in a test tube and stored for no more than one hour. Each test tube contained 20 ml icecold Krebs-Ringer bicarbonate solution, in which the slices were allowed to circulate freely. The salt solution, which had the following composition, meq./l. Na 144 K^+ 4.7; Ca^{++} 5.0; Mg^{++} 2.4; Cl^- 130; HCO_3^- 25; $H_2PO_4^-$ 1.2, contained glucose (2 g/l). Incubations were carried out by shaking (60 oscillations/min.) the test tubes at 37° and *bubbling a 5:95 mixture of either carbon dioxide-oxygen or carbon dioxide-nitrogen* through the medium.

The pH of the salt solution aerated with either mixture was 7.4 as determined (5 experiments) by a pH meter (model 27 Radiometer Copenhagen). The slices were preincubated for 10 minutes previous to the addition of ^{14}C-decamethonium or ^{14}C hexamethonium. Approximately the same amount of radioactive material (0.2 µcl) was used in each of the experiments and the desired concentration of the compound was always obtained by adding unlabelled carrier. Potential inhibitors were added to the media just before ^{14}C-decamethonium.

Analytical methods.

At the end of the incubation period the slices were separated from the media by filtration on cotton wool, weighed and prepared according to the method employed by Brøchner Christensen (1965) for the analysis of tissue samples. An aliquot of 1000 µl of th medium was prepared by the same procedure as that described for the analysis of plasma samples (Brøchner Christensen 1965). The radioactivity of each sample was measured by means of a Packard Tri-Carb liquid scintillation spectrometer model 314 EX. Counting rates were at least 10 times the background counting rate, and not less than 5000 counts were recorded. The counting efficiency was measured by adding 25 µl f internal standard (toluene-^{14}C with a disintegration rate of 4.36 × 10^5 dpm/ml from the New England Nuclear Corp.) to pairs of corresponding tissue and medium samples. The ratio between counting rates recorded from each pair of samples was 1.00 ± 0.01 (the mean ± S. E. M. of values from 41 experiments) indicating that the two types of samples were counted with the same efficiency. The recovery of known amounts of ^{14}C-*decamethonium added to blank medium and tissue specimens*, which were then subjected to the standard procedure, constituted 102.3 ± 1.7 % in the medium samples and 108.4 ± 1.7 % in the tissue samples (mean ± S. E. M. of values from 6 experiments).

The results were expressed as a slice-to-medium (S/M) concentration ratio of ^{14}C calculated as radioactivity per g slice (wet weight)/radioactivity per ml medium. In some experiments the uptake of ^{14}C-decamethonium by the kidney slices (µmol per kg tissue) was calculated from the S/M ratio and the ^{14}C-decamethonium concentration of the medium (µmol per l medium). As the volume of the medium was about hundred times larger than that of the slices the external ^{14}C-concentration could be considered as being practically constant during the incubation period.

Experiments were carried out for the purpose of estimating to what extent slices *swelled or lost* solid matter during incubation. The total content of tissue water which was determined by drying the tissue at 105 for 24 hours, was expressed as per cent of wet weight. The water content of freshly prepared slices was 75.7 ± 1.5 % whereas

that of slices incubated aerobically for one hour was 80.0 ± 0.7 % (mean values ± S. E. M. from 6 experiments). The difference between the two mean values is significant ($P < 0.05$).

Preparation of chromatograms.

Following incubation with ^{14}C-decamethonium protein-free kidney extracts were prepared as described (BROWN CHRISTENSEN 1965) and chromatographed on paper by the method previously described (BROWN CHRISTENSEN & HOLM 1969). Two different systems were used at the mobile phase: A, n-butanol, ethanol, glacial acetic acid, water (8.2:1.3). B, pyridine, n-butanol, water (3.2.3).

Results

Decamethonium uptake and duration of incubation.

Fig. 1 shows the relationship between the uptake (S/M concentration ratio) of ^{14}C-decamethonium (1 8 μM) by mouse kidney slices and the duration of incubation. The upper curve represents the uptake in an atmosphere

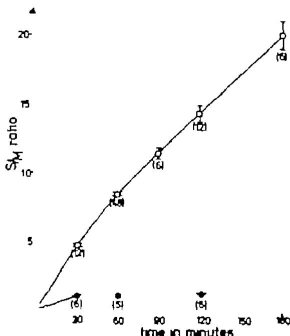


Fig. 1 ^{14}C-decamethonium uptake by mouse kidney slices in oxygen (○) or nitrogen (●) atmosphere. The slice/medium concentration ratio of decamethonium is plotted against duration of incubation (minutes). The decamethonium concentration of the medium was 1.8 μM in all experiments. The circles represent mean values with S. E. M. (vertical bars) and figures in brackets indicate number of experiments.

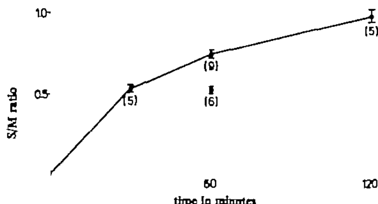


Fig. 2. [14]C-hexamethonium uptake by mouse kidney slices in oxygen (○) or nitrogen (●) atmosphere. The slice/medium concentration ratio of hexamethonium is plotted against duration of incubation (minutes). The hexamethonium concentration of the medium was 2.0 μM in all experiments. The circles represent mean values with S. E. M. (vertical bars) and figures in brackets indicate number of experiments.

of oxygen and shows that the S/M ratio which approximates 9 after one hour continues to increase almost linearly during three hours of incubation. The lower curve representing the uptake in an atmosphere of nitrogen shows that the S/M ratio reaches a constant value of about one, within half an hour

The size of the extracellular space of the slices was estimated by determining the S/M concentration ratio (one hour of incubation) of [14]C-sucrose (1.2 μM), which was 0.52 ± 0 01 in oxygen and 0.59 ± 0 01 in an atmosphere of nitrogen (mean values ± S. E. M. from six experiments). The difference between the two mean values is significant ($P < 0\ 01$).

Hexamethonium uptake and duration of incubation.

The curve in fig. 2 shows the relationship between S/M concentration ratio and duration of incubation. Kidney slices were incubated with 2.0 μM [14]C-hexamethonium in an atmosphere of oxygen. It is seen from the course of the curve that the S/M ratio which attained a value of 0.7–0.8 after one hour of incubation, increased slowly with time approximating to unity after two hours. In an atmosphere of nitrogen the S/M ratio was 0.5 after one hour of incubation.

Decamethonium uptake and external concentration.

Fig. 3 shows the relationship between the decamethonium uptake (μmol/kg tissue)) after one hour of incubation and the concentration of ^{14}C-decamethonium in the medium. The upper curve represents the uptake in an atmosphere of oxygen, while the uptake in nitrogen is represented by the lower curve It is seen that the uptake under anaerobic conditions is practically directly proportional to the external concentration, whereas this is not the case under aerobic conditions. Thus, the aerobic uptake seems to consist of at least two components, i. e. one that becomes saturated with increasing decamethonium concentration in the medium and one that is linear with the external concentration.

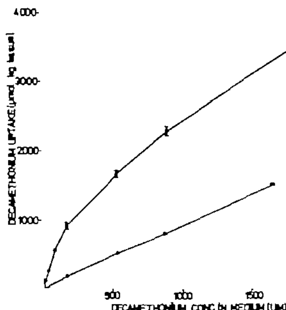


Fig. 3. Relationship between the uptake of ^{14}C-decamethonium (μmol/kg tissue) by mouse kidney slices (incubation period one hour) and the concentration of decamethonium in medium. The uptake was determined in both oxygen (○) and nitrogen (●) atmosphere. Each point is the mean of values from 5–12 experiments. The vertical bars indicate S. E. M.

The uptake was calculated from the S/M ratio and the concentration (μM) of ^{14}C-decamethonium in the medium.

The influence of metabolic conditions on the uptake of decamethonium.

The S/M concentration ratio (incubation period one hour) of [14]C-decamethonium (1 8 μM) was determined in an atmosphere of oxygen under various metabolic conditions.

Table 1 shows that the S/M ratio of decamethonium is depressed by about 85 / in the presence of 500 μM 2,4-dinitrophenol or 1000 μM iodoacetate, whereas it is not affected by ouabain (10 μM) The S/M ratio was reduced by 82 / when incubations were carried out at 24

The influence of quaternary ammonium compounds on the uptake of decamethonium

The S/M concentration ratio (incubation period one hour) of [14]C-decamethonium (1 8 μM or 18 μM) was determined in an atmosphere of oxygen in the presence of various quaternary ammonium compounds.

It is seen in table 2 that the S/M ratio of decamethonium (1.8 μM) was depressed by 36 / in the presence of 1000 μM hexamethonium, whereas this was not the case with a tenfold lower concentration of hexamethonium. The inhibitory action of 1000 μM hexamethonium was reduced from 36 / to 12 / following a tenfold increase in decamethonium concentration. The addition of 1000 μM tetraethylammonium to the medium caused a reduction in the S/M ratio of decamethonium (1 8 μM) of 22 / but a similar effect was not observed with 2000 μM N[1]-methylnicotinamide.

Paper chromatographic results

Protein free extracts from kidney slices, incubated for one hour in an atmosphere of oxygen with [14]C-decamethonium (1 8 μM) were subjected to paper chromatography The radioactivity of the chromatograms could not be distinguished from that of authentic [14]C-decamethonium.

Table 1

The uptake (slice/medium concentration ratio) of decamethonium in the presence of metabolic inhibitors and at 24 Mouse kidney slices were incubated for one hour in oxygen atmosphere with 1.8 μM [14]C-decamethonium. Figures in brackets indicate μmolar concentration f inhibitor Results are given as the mean ± S. E. M. of values from 6 experiments*).

Treatment	S/M ratio
None —	8.57 ± 0.15
No heating, 24	1.54 ± 0.03
Ouabain (10)	8.74 ± 0.15
2,4-dinitrophenol (500)	1.30 ± 0.02
Iodoacetate (1000) —	1.23 ± 0.02

) 48 control experiments.

Table 2

The influence of quaternary ammonium compounds on the uptake (slice/medium concentration ratio) of ^{14}C-decamethonium (1.8 μM or 18 μM) by mouse kidney slices incubated for one hour in oxygen atmosphere. Figures in brackets indicate μmolar concentration of various agents. Results are given as the mean ± S. E. M. of values from 6 experiments*).

Treatment	S/M ratio	
	1.8 μM decamethonium	18 μM decamethonium
None	8.57 ± 0.15	7.40 ± 0.25
Hexamethonium (1000)	5.47 ± 0.08	6.48 ± 0.22
Hexamethonium (100)	9.33 ± 0.28	
Tetraethylammonium (1000)	6.68 ± 0.23	
N[1]-methylnicotinamide (2000)	9.02 ± 0.51	

) 48 control experiments with 1.8 μM decamethonium.

Discussion

The present experiments show a significant accumulation of decamethonium by mouse kidney slices, whereas the concentration of hexamethonium in the slices do not exceed that of the medium. There is also a considerable difference between these two methonium compounds regarding their renal uptake in other mammals. Experiments with rat and cat kidney slices (McIsaac 1969) show that ^{14}C-decamethonium is accumulated in the rat kidney whereas this is not the case with ^{14}C-hexamethonium. However in contrast to the conditions in mice and rats, there is a considerable accumulation of ^{14}C-hexamethonium and no accumulation of ^{14}C-decamethonium in the cat kidney

Under aerobic conditions and at a low external concentration, decamethonium is taken up by mouse kidney slices at a practically constant rate and against an increasing tissue-to-medium concentration gradient, no equilibrium between uptake and loss being attained within 3 hours of incubation. This uptake kinetics might be due to a binding of decamethonium to tissue components, as the latter process would maintain a concentration gradient between unbound decamethonium in tissue and medium. The very high renal ^{14}C-concentrations even four hours after the intravenous injection of ^{14}C decamethonium into mice (Broen Christensen & Holm 1969) are consistent with a firm binding of decamethonium in the kidney tissue. A similar uptake kinetics was observed by McIsaac (1965 & 1969) whose experiments showed that kidney slices from cats and rats continued to accumulate ^{14}C-hexamethonium and ^{14}C-decamethonium respectively for several hours, without

attaining of equilibrium between uptake and loss. Furthermore, *in vivo* results (McIsaac 1962) *showing particularly high and increasing concentrations* of ^{14}C hexamethonium in the kidneys for several hours following a single intravenous injection into cats, are indicative of a rather firm binding of hexamethonium in the cat kidney Autoradiographic studies showed that the radioactivity was primarily localized in the tubule cells of the cortex. Although ^{14}C hexamethonium was concentrated in the kidneys of intact cats in up to 40–70 times the plasma concentration, McIsaac (1962) did not find any evidence for a tubular secretion of this compound, as the hexamethonium/creatinine clearance ratio was 0 92

The accumulation by kidney slices of the monoquaternary compounds N^1-methylnicotinamide and tetraethylammonium exhibits kinetics essentially different from those described above for the accumulation of bisquaternary compounds. Thus, studies with N^1-methylnicotinamide in cats (McIsaac 1965) and tetraethylammonium in rats (McIsaac 1969) showed that equilibrium *between uptake and loss was already reached within two hours of* incubation. We have no information about the renal elimination of N^1-methylnicotinamide and tetraethylammonium in cats and rats. However N^1-methylnicotinamide (Beyer *et al* 1950) and tetraethylammonium (Rennick *et al.* 1954) are rapidly secreted by kidney tubules in dogs. Furthermore, the accumulation of N^1-methylnicotinamide by dog kidney slices (Farah *et al* 1959) exhibits kinetics completely similar to those just described for the accumulation of N^1-methylnicotinamide by cat kidney slices

In an attempt to *explain these differences* between bis- and monoquaternary compounds McIsaac (1965 & 1969) suggested that bisquaternary ammonium compounds penetrate into the tubular cells bound to a carrier involved in the renal tubular transport of organic bases but in contrast to the rapidly secreted monoquaternary compounds they are subsequently strongly bound to nonspecific intracellular sites, which make them unavailable for rapid secretion into the tubular lumen. This hypothesis is supported by *in vitro* results showing that both tetraethylammonium and decamethonium inhibit the renal accumulation of hexamethonium in cats (McIsaac 1965) Furthermore, the renal uptake of decamethonium in rats (McIsaac 1969) as well as that of hexamethonium in cats require energy as both processes are markedly depressed by metabolic inhibitors.

Hence, it is possible that tubular transport processes similar to those suggested by McIsaac ((1965 & 1969) participate in the renal accumulation of decamethonium in mice. Within the concentration range studied the aerobic uptake consists of at least two components, i. e. one that is saturable and one that is linear with the decamethonium concentration in the medium. These kinetics are most simply explained by assuming the presence of two distinct mechanisms for the uptake of decamethonium, one mediated by a

carrier the other by a passive diffusion into the cells through pores. The almost complete inhibition of the decamethonium accumulation under anaerobic conditions, at 24 and in the presence of 2,4-dinitrophenol and iodoacetate indicates the involvement of energy requiring processes in the uptake. The anaerobic uptake which was directly proportional to the decamethonium concentration of the medium, exceeded the size of the extracellular space. This supports the suggestion that decamethonium penetrates into the cells partly by passive diffusion. Both hexamethonium and tetraethylammonium depressed the decamethonium uptake, which suggests that bis- and monoquaternary ammonium compounds combine in part at least, with a common carrier N^1-methylnicotinamide at a very high concentration did not inhibit the uptake and consequently the affinity of this agent to a possible carrier should be very low as compared to that of decamethonium. The experimental data do not allow any conclusions to be drawn concerning the type of inhibition exhibited by these agents, but the fact that the inhibitory action of hexamethonium is considerably decreased by a tenfold increase of the decamethonium concentration is consistent with competition for a common carrier The weak inhibition of the decamethonium uptake seen with hexamethonium and the insignificant uptake of the latter suggests that hexamethonium has a low affinity to the postulated transport system. In an unpublished whole-body autoradiographic study of the distribution of ^{14}C decamethonium we attempted to determine whether the cortical or medullary areas of the mouse kidney were the most active in binding decamethonium. Following the intravenous administration of ^{14}C-decamethonium to a mouse, the highest concentration of radioactivity was found in the kidney particularly in the cortex.

In vitro studies (Brøen Christensen 1967), showing that the accumulation of ^{14}C-decamethonium by mouse liver slices was an energy dependent process inhibited by hexamethonium, support the assumption that qualitatively identical mechanisms are important for the renal as well as for the hepatic accumulation of decamethonium in mice. Furthermore, the very high hepatic ^{14}C-concentrations four hours after the intravenous injection of ^{14}C-decamethonium into mice (Brøen Christensen & Holm 1969) are indicative of a firm binding of decamethonium in the mouse liver

Acknowledgements

I sincerely thank cand. scient. Aksel Jørgensen, who kindly performed the autoradiographic study The Packard Tri-Carb Scintillation spectrometer of the department used in the study was purchased with a grant given by the Danish State Research Foundation (A 5/65) Mrs. Karin Jørgen-Jensen and Mr Bjarne Lauritzen gave technical assistance.

REFERENCES

Beyer K. H., H. F Russo, S. R. Gass, K. M. Wilhoyte & A. A. Pitt. Renal tubular elimination of N[1]-methylnicotinamide. *Amer J Physiol.* 1950, 160, 311 320.

Broen Christensen, C.. Distribution and biliary excretion of decamethonium in doubly nephrectomized rabbits. *Acta pharmacol. et toxicol.* 1965, 23 275–286.

Broen Christensen, C.. Decamethonium uptake by slices of mouse liver *Biochem. Pharmacol.* 1967 16 2221–2222.

Broen Christensen, C. & Jan Holm. The distribution and urinary excretion of decamethonium and hexamethonium following intravenous injection into mice. *Acta pharmacol et toxicol.* 1969 27 17–26.

Farah, A., M. Frazer & E. Porter Studies on the uptake of N1-methylnicotinamide by renal slices of the dog. *J Pharmacol.* 1959 126, 202 211.

McIsaac, R. J The relationship between distribution and pharmacological activity of hexamethonium-N-methyl-^{14}C. *J Pharmacol.* 1962, 135 335–343

McIsaac, R. J The uptake of hexamethonium-C^{14} by kidney slices. *J Pharmacol.* 1965, 150 92–98.

McIsaac, R. J The binding of organic bases to kidney cortex slices. *J Pharmacol.* 1969, 168, 6–12.

Rennick, B. R., D. M. Calhoun, H. Gandia & G. K. Moe: Renal tubular secretion of tetraethylammonium in the dog and chicken. *J Pharmacol.* 1954 110, 309–314.

Acta pharmacol. et toxicol 1970 28 203–208

From the Section of Experimental Anaesthesiology (Professor O. Secher M. D.) and the Department of Pharmacology (Professor J. Schou, M. D.), University of Copenhagen, Denmark

The Protective Action of Different Barbituric Acid Derivatives against Anoxia in Mice

By

Bendt J. Wilhjelm and E. Jacobsen

(Received November 3, 1969)

Abstract: The protective action of eight different barbituric acid derivatives against anoxia was investigated in mice. The increase in survival time ranged between 0 % and 180 %. Three drugs was given in both equimolar and equipotent doses; for these drugs the increase in survival time ranged between 50 % and 180 % indicating that the difference in protective action depends on differences between the miscellaneous barbituric acid derivatives.

Key-words: Anoxia – barbiturates.

Previous studies have shown that the survival of hypoxic mice previously anaesthetized with thiomebumalum (NFN) (= thiopental (WHO USP) = thiopentone (BP) = pentothal®) is longer than that of unanaesthetized mice exposed to the same low oxygen concentration (ARNFRED & SECHER 1962, WILHJELM & ARNFRED 1965).

Halothanum (NFN BP) (= fluothane®), cyclopropanum (NFN BP), urethan, hydroxydion (WHO NFN) (= viadril®) and 2-methoxy-allylphenoxyacetic acid-N N-diethyl-amide (= detrovel®) have been shown to have a similar protective action (WILHJELM & ARNFRED 1965 WILHJELM 1965).

Chloroform (chloroformium ad narcosin (NFN)) trichloroethylene (= trichloraethylenum ad narcosin (NFN) = trilene®) and methoxyflurane (= methoxyfluranum (NFN, WHO) = pentrane®) had less protective effects and neither ether (aether ad narcosin (NFN)) nor nitrous oxide (nitrogenii monoxidum (NFN)) could be shown to have any effect (WILHJELM & ARNFRED 1965).

Some anaesthetic agents cause a reduction in the oxygen uptake by cerebral tissue (QUASTEL & WHEATLEY 1932). This leads to the hypothesis, supported by the results of ARNFRED & SECHER (1962) and WILHJELM (1965), that the depressant effect of thiomebumalum on cerebral oxygen consumption causes

Table 1

The barbituric acid derivatives mentioned in the text.

NFN name	WHO name and other names	Registered trademark	Chemical constitution
Enallynymalum	Methohexitalum (INN US) Methohexitone (BP)	Brevital Brietal	5-ally 1-methyl-5-(1-methylpentyn-(2)-yl)-barbituric acid
Diemalum	Barbitalum (INN US) Barbitone (BP) Diemal	Medinal Veronal	5,5-diethyl-barbituric acid
Phenemalum	Phenobarbitalum (INN US) Phenobarbitone (BP) Fenemal	Adonal Bardiphen Gardenal Luminal Molinal Starifon	5-ethyl-5-phenyl-barbituric acid
Pentymalum	Amobarbitalum (INN US) Amylobarbitone (BP)	Amydorm Amytal Barbamyl Dormytal Eunoctal Isomyl	5-ethyl 5-isoamyl-barbituric acid
Allypropymalum	Amobarbitalum (INN US) Allypropymal	Alurate Isonal Numal	5-allyl-5-isopropyl-barbituric acid
Enhexymalum	Hexobarbitalum (INN, US) Hexobarbitone (BP) Enhexymal Enimal	Citodon Cyclonal Evipan Noctivane Privenal Tobinal	5-cyclohexen-(1)-yl-1,5-dimethyl-barbituric acid
Enibomalum	Enibomal	Ennarcon Narcotal Narcodorm	5-(2-bromallyl)-5-isopropyl 1 methyl-barbituric acid
Mebumalum	Pentobarbitalum (INN US) Pentobarbitone (BP) Mebumal	Barbityral Nembutal Sombutal	5-ethyl-5-(1-methyl butyl)-barbituric acid
Thiomebumalum	Thiopentalum (INN, US) Thiopentone (BP)	Leopental Pentothal Tio-pentamal	5-ethyl-5-(1-methyl-butyl)-2-thio-barbituric acid

a greater resistance to hypoxia in animals anaesthetized with thiomebumalum than in non-anaesthetized animals.

In previous studies (ARNFRED & SECHER 1962 WILHELM & ARNFRED 1965) thiomebumalum was the only barbituric acid derivative used. As a continuation of these investigations, the properties (related to anoxia) of a series of commercially available injectable barbituric acid derivatives were investigated. The barbituric acid derivatives under consideration are listed in table 1 by their NFN WHO and other names, together with some of their registered trademarks and chemical structure.

Methods

The experimental animals were male white mice of a single strain (Leo Stritt) weighing from 25 to 40 g.

The experimental procedure has been described previously (WILHELM & ARNFRED 1965).

The barbituric acid derivatives were administered intraperitoneally in equimolar concentrations (0.0368 molar solution was used in all experiments) in doses cor responding to 55 mg thiomebumalum per kg mice.

When asleep, or at least 20 minutes after the injection, the animals were placed in bottles contained in a thermostatically controlled box. A mixture of 20 per cent oxygen and 80 per cent nitrogen was perfused through the bottles for five to ten minutes after the mice had been placed in them. The concentrations were then altered to 5 per cent oxygen and 95 per cent nitrogen. The percentages are /v

The action of each barbituric acid derivative was observed during experiments on 50 mice, i.e. 25 anaesthetized and 25 control animals. These experiments were conducted as five duplicated pairs. Each of these involved five anaesthetized and five non-anaesthetized mice, both groups being exposed to the same low oxygen concentration.

The oxygen concentration in the five individual bottles and at the inlet and outlet of the bottles was checked with a Radiometer® oxygen electrode. No difference in oxygen percentage was observed.

The survival time, i.e. the interval between the reduction of the oxygen concentration to 5 per cent and the cessation of respiration was measured for each mouse in both groups.

Results

The survival time of mice anaesthetized with the various barbiturates and those in the corresponding control groups are listed in table 2. The average prolongation of the survival time varies between 9.5 and 0 0 minutes, and the average percentage prolongation varies between + 188.4 per cent and − 1 4 per cent. A statistical analysis of the material gave no significant prolonga tion of the survival time for enallynymalum or diemalum. A significant prolongation of survival time was demonstrated for pentymalum ($P < 0\ 005$) and for phenemalum, allypropymalum, enhexymalum, enibomalum and mebumalum ($P < 0\ 001$).

Table 2

Average survival time in minutes for control mice and anaesthetized mice, with percentage prolongation in survival time.

Average survival of control mice (min.)	S. E. M.	Anaesthetic (NFN names)	Average survival of anaesthetized mice (min.)	S. E. M.	Increase of survival time (%)
4.3	0.2	Enallynymalum I	4.2	0.5	−1.4
5.0	0.4	Enallynymalum II	5.0	0.4	1.6
4.6	0.3	Diemalum	4.8	0.3	4.3
4 4	0.2	Phenemalum	6.4	0.4	47 7
4 8	0.3	Pentymalum	7.5	0 8	56.4
4.9	0.2	Allypropymalum	10.0	0.8	106 2
5.2	0.4	Enbexymalum	12.5	1.3	142.6
4.6	0.2	Enibomalum	11 4	1.2	147.8
5.0	0.3	Mebumalum	14 4	1.9	188.4

Discussion

We have allotted the barbituric acid derivatives investigated in this series to one of three groups according to their protective action against hypoxia in mice. Mebumalum, enibomalum, enbexymalum and allypropymalum extend the survival time between 100 and 200 per cent. Thiomebumalum, which extended the survival time to 154 per cent, belongs to the same group (WILHJELM & ARNFRED 1965) Phenemalum and pentymalum add about 50 per cent to the survival time, while diemalum and enallynymalum give little or no protection. The hypnotic action of enallynymalum is of short duration. Presumably its effect ceases before severe hypoxia develops.

There is a considerable protective action by mebumalum against anoxia and no protective action by diemalum in agreement with the investigations of ZORN *et al.* (1939) These authors examined the inhibition of various barbituric acid derivatives on the oxygen uptake of rat liver slices. Diemalum in amounts from 0 001 to 0 1 g per 100 g of liver tissue did not reduce the oxygen uptake. Other barbituric acid derivatives caused inhibition of liver oxygen uptake in proportion to their concentrations. Pentymalum and mebumalum gave maximum inhibition while allypropymalum and enbexymalum had only slight effects. This latter finding is contrary to our own observations, but may be explained by dose variation.

The animals in our control groups died in convulsions, as did the anaesthetized animals, but in most cases the onset was later in the anaesthetized group. The later appearance of convulsions may explain the small extention of the survival time seen after pentymalum and phenemalum, but not the

considerable increase in survical time produced by mebumalum, enibomalum and enhexymalum. According to the results of previous studies (WILHJELM & ARNFRED 1965) the prolongation of survival time that is due to the prevention of death in convulsions of anaesthetized mice not in anoxia is considered to range between 8 and 12 / The mice anaesthetized with enibomalum twitched throughout the anoxic period nevertheless the survival time increased by 148 per cent.

Some physico-chemical properties of the drugs under consideration are compiled in table 3 in an attempt to correlate the survival time with one or more of these properties.

High pKa-values give a higher concentration of the active undissociated acid at body pH, but there was no relation between high pKa-values and long survival time. In similar experiments with anoxic mice, WILHJELM & LANGGÅRD (unpublished) found a change in pH from 7.33 to 7 13 A decrease in pH of this size however will add little to the activity of the barbituric acid derivatives.

Table 3

Physico-chemical properties of barbituric acid derivatives.

Increase of survival time (%)	Barbituric acid derivatives (NPN-names)	Molecular weight	pK	Lipid solubility*	Anaesthetic (AD50) dose in mice in mg
−1 4	enallynymalum I	262.3	8.2	1000	12 L
1.6	enallynymalum II				
4.3	diemalum	208.2	7.8	1	260 L p.
47 7	phenemalum	232.2	7.3	3	134 L p.
56.4	pentymalum	226.3	7.8	42	65 L p.
106.2	allypropymalum	210.2	8.0	–	–
142 6	enhexymalum	236.3	8.2	290	75 p.
147.8	enibomalum	303.2	8.2	–	–
188.4	mebumalum	226.3	8 0	59	60 L p.
134 0	thiomebumalum	242.3	7 4	580	

Partition coefficient at app. 25° of the unionized form between methylene chloride and water (BUSH 1963).

** BARNES & ELTHERINGTON (1966).

The lipid solubility of barbituric acid derivatives determines the time which elapses before maximum brain concentrations are reached. Lipid solubility is dependent on the side chain at carbon atom 5 – a longer side chain increases the solubility of the drug in non-polar solvents. The hypnotic effect of a given drug is also related to the length of the side chain. Our investigation seems to indicate that there is no relationship between the lipid solubility of the different barbituric acid derivatives and their protective action against anoxia.

We administered our barbituric acid derivatives in equimolar doses as the equivalent of 55 mg thiomebumalum per kilo mouse. It would appear (table 3) that the mean anaesthetic dose (AD50) varies considerably for most of the barbituric acid derivatives used in our experiments.

The animals given phenemalum and diemalum were awake during the anoxic exposure, which may be one of the reasons for the modest increase in survival time observed after the administration of these drugs.

The AD50 of pentymalum and enhexymalum and mebumalum are almost identical, and the animals treated with these drugs not only received an equimolar dose, but also at the same time an equipotent dose. The increase in survival time for these barbituric acid derivatives varied from 56.4–188.4 / It is possible that this is due to the fact that the different barbituric acid derivatives possess different protective actions against anoxia.

REFERENCES

Arnfred, I. & O. Secher Anoxia and barbiturates. *Arch. int Pharmacodyn.* 1962, 139 64–74

Barnes, C. D & L. G Eltherington. *Drug dosage in laboratory animals* University of California Press. Berkeley and Los Angeles, 1966.

Bush, M T Sedatives and hypnotics. In. *Physiological pharmacology Vol. I* Root, W S. & F G Hofmann (Eds.) Academic Press, Inc., New York 1963.

Quastel, J H. & A. H. M. Wheatley Narcosis and oxidation of the brain. *Proc. R. Soc. Biol.* 1932, 112, 60–79

Wilhjelm, B. J & I. Arnfred. Protective action of some anaesthetics against anoxia. *Acta pharmacol. et toxicol.* 1965 22, 93–98.

Wilhjelm, B. J Further investigations into the protective action of anaesthetics against anoxia in mice. *Act pharmacol et toxicol.* 1965, 22, 131–134.

Zorn, C. M., E. Muntwyler & O. W Barlow The effect of certain barbiturates upon the oxygen uptake and anaerobic reduction of methylene blue by rat liver and brain. *J Pharmacol. Exp Ther* 1939 66, 326–355

Acta pharmacol. et toxicol. 1970 **28**, 209–214

Department of Pharmacology Karolinska Institutet,
Stockholm, Sweden

The Plasma Protein Binding of Amphetamine, Catecholamines and Related Compounds

By

Gunhild Franksson and Erik Änggård

(Received December 5 1969)

Abstract. The plasma protein binding of amphetamine, phenmetrazine, adrenaline, noradrenaline, dopamine, normetanephrine and metanephrine was studied using equilibrium dialysis with human plasma. The binding of amphetamine and phenmetrazine at 5×10^{-7}M to 5×10^{-4}M was about 20 %. No difference in the binding of amphetamine was found between drug non dependent and tolerant dependent subjects. At physiological concentrations ($< 10^{-6}$M) adrenaline was bound to about 20 % noradrenaline and dopamine to about 13 %. The binding of metanephrine and normetanephrine was of lower order i.e. about 5 %. The results are of value for the understanding of the overall pharmacodynamic situation in amphetamine dependence.

Key-words: Amphetamine – phenmetrazine – catecholamine – blood proteins.

The intravenous self-administration of central stimulants is accompanied by the rapid development of tolerance to the drug as well as by psychotic manifestations such as paranoid delusions and stereotyped behaviour (Connell 1958 Kalant 1966 Sjöqvist & Tottie 1969 Garattini & Costa 1969). The increased tolerance could be due to alterations in the distribution, metabolism and elimination of the drug as well as to induced biochemical changes at the receptor level in the adrenergic neurons. The present report dealing with the plasma protein binding of a series of sympathomimetic amines, is part of a study on the pharmacodynamics and pharmacokinetics of amphetamine in chronically dependent human subjects.

Material and Methods

The binding experiments were conducted on a pool of plasma drawn from blood donors at the Blood Bank at Karolinska sjukhuset. Citrate 0.015 M or heparin were

used as anticoagulants. In preliminary experiments no difference was observed between the binding properties of citrate and heparin plasma.

For comparison of the binding of amphetamine in dependent and non drug dependent subjects, 10 ml of plasma was collected under sterile conditions in heparinized tubes. The 6 abusers had previously been admitted in a state of amphetamine psychosis and had histories of prolonged (> one year) intravenous use of amphetamine in high doses. The subjects were between 16 and 25 years of age. The samples were taken when no amphetamine was detectable in the urine and plasma. Generally this occurred on the 3rd to 5th day after admission. The 6 non dependent control subjects were selected from the laboratory personnel of the same age group.

^{3}H labelled amphetamine sulphate and phenmetrazine (phenmetralinum NFN) hydrochloride were prepared by acid catalytic exchange (New England Nuclear Corp Boston, Mass.). The specific activity of the amphetamine sulphate was 9 ci/mmole and that of the phenmetrazine hydrochloride 320 mci/mmole. Radiopurity was controlled by thin layer chromatography and found to be > 98 %. With both compounds exchange of tritium for protons in the medium occurred at rate of a few percent per month. Prior to these experiments the ^3H_2O was removed by freeze drying.

DL-adrenaline – 7^{3}H, 6.8 ci/mmole, radiopurity 99 % DL-normetanephrine – 7^{3}H, about 3 ci/mmole, DL-metanephrine 10 ci/mmole, radiopurity 99 % and dopamine ^{3}H (generally labelled), 10 ci/mmole were supplied by the New England Nuclear Corp. DL-noradrenaline – 7^{3}H hydrochloride, 3.6 ci/mmole, radiopurity 95 % was obtained from the Radiochemical Centre, Amersham, Bucks., England. Dilutions to the desired specific activity were achieved by the addition of the corresponding unlabelled drugs.

The plasma protein binding of the amines was studied by means of equilibrium dialysis (SCHACH VON WITTENAU & YEARY 1963). Ultrafiltration using Amicon UM-2 and PM 10 filters (BIXLER 1968) was found to be unsatisfactory for binding studies since considerable amounts of the amines became bound to the membrane. The drugs were dissolved in water and added t the plasma in a volume not exceeding 0.1 ml. The plasma volume was 10 ml. The tubing (Union Carbide no. 376 flat diam. 24 mm) was cut into 20 cm pieces and secured with knots placed 9 cm from each other. The excess tubing was cut off. The bags were put into 110 × 24 mm test tubes containing 11 ml of 0.1 M potassium phosphate buffer pH 7.4. With this amount of buffer equal volumes on both sides of the dialysis membrane were found at equilibrium. The tubes were put in a rack and rocked gently at room temperature for period of 16 hours. Equilibrium under these conditions was attained within eight hours. In each experiment controls with buffer substituted for plasma were included in order to check that complete equilibrium had occurred. To prevent microbiological growth merthiolate ® was added to the plasma in a final concentration of 0.5 mM. This addition had no effect on the protein binding of the drugs under study.

At the start of the experiment each drug was examined in triplicate in concentrations in the plasma of 10^{-6}M, 2×10^{-6}M 10^{-5}M, 2×10^{-5}M and 10^{-4}M. At equilibrium the outside volume was measured and three 100 µl aliquots from each side were pipetted into liquid scintillation vials. For the analysis of the nonpolar amines 50 µl of 2 N sodium hydroxide was added together with 10 ml of toluene scintillation solution. With the phenolic amines 10 ml of counting solution based on cellosolve was used. The protein present in the aliquot from the inside had no effect on the counting efficiency. The amount of bound drug was calculated from the formula.

$$\text{binding } \% = \frac{\text{counts inside} - \text{counts outside}}{\text{counts inside}} \times 100.$$

Results

Binding of amphetamine and phenmetrazine.

The results on the binding of amphetamine and phenmetrazine to the plasma proteins are summarized in table 1 Both amphetamine and phen-

Table 1

Binding of sympathomimetic amines to plasma proteins.

Drugs	Conc. (10^{-7}M)	% bound Mean and range
Amphetamine	5.2	21 (8)
	10.4	22 (9)
	52	22 (2)
	104	24 (1)
	520	1 (2)
Phenmetrazine	5.3	21 (3)
	10.5	20 (2)
	52	16 (14)
	105	10 (5)
	530	21 (1)
Adrenaline	5.3	23 (7)
	10.3	20 (12)
	49	7 (5)
	98	5 (13)
	490	7 (9)
Noradrenaline	5.1	12 (11)
	10.2	13 (6)
	48	2 (17)
	96	3 (2)
	490	4 (4)
Dopamine	5.1	14 (6)
	10.2	13 (1)
	50	8 (9)
	100	8 (7)
	500	8 (6)
Metanephrine	4.8	3 (4)
	9.8	5 (3)
	49	5 (7)
	98	6 (3)
	490	7 (6)
Normetanephrine	5.9	5 (9)
	9.6	0 (4)
	48	1 (6)
	96	1 (5)
	480	3 (12)

Table 2

Comparison between the plasma protein binding of amphetamine in dependent and drug naive subjects. The concentration of amphetamine was 10^{-6}M.

Subject	Age	Sex	Dependent	% bound amphetamine	Mean ± S. E. M.
I. K.	24	♀	−	25	
B. E.	21	♀	−	25	
K. E.	23	♂	−	23	
L. Ö.	31	♀	−	20	
B. R.	23	♀	−	19	
L. J.	27	♂	−	25	23 % ± 1.1
K. L.	16	♂	+	26	
M. L.	19	♀	+	28	
B. H.	15	♀	+	30	
G. N.	25	♂	+	26	
H. W.	24	♂	+	24	
G. S.	23	♀	+	23	26 % ± 1.0

$P > 0.05$

metrazine were bound to about 20 %. No significant difference was noted in the concentration range studied. Moreover no difference in the binding of amphetamine was found between the plasma from tolerant amphetamine dependent subjects and from drug non dependent subjects (table 2).

Binding of phenolic amines

The plasma protein binding was studied for adrenaline, noradrenaline, dopamine, metanephrine and normetanephrine. The results are shown in table 1. In physiological concentrations adrenaline was bound to 23 %, noradrenaline to 12 % and dopamine to 14 %. With higher concentrations of these amines, the binding decreased to a few percent. Metanephrine and normetanephrine were not significantly bound even in the lowest concentration.

Discussion

Our results show that amphetamine and phenmetrazine in concentrations likely to be found in plasma after doses of 0.02 to 0.2 g given intravenously (Änggård *et al.* unpublished) are not appreciably bound to the plasma proteins. Marked species differences in the degree of plasma protein binding of basic drugs have been described (Borgå *et al.* 1968). In spite of this, our

data agree well with AXELROD's (1954) early finding that in the dog 15 % of the amphetamine is bound at the 2–10 μg/ml concentration. The low degree of binding of amphetamine and phenmetrazine therefore makes it unlikely that these drugs would exhibit any interaction with other drugs by a displacing effect.

No difference in binding of amphetamine was noted between the plasma from tolerant dependent subjects and from non dependent subjects. These data then rule out the possibility that part of the tolerance seen in amphetamine dependence could be due to an increased binding capacity of the plasma proteins.

Few studies have been made on the plasma protein binding of basic drugs. In this connection it might be noted that another group of nonpolar amines, the tricyclic antidepressants show a binding of more than 90 % (BORGÅ *et al.* 1969). It is possible that the presence of the large tricyclic ring system may interact more closely with the protein by short range secondary molecular forces, and that the amino group in the basic drugs therefore plays a minor role in the binding.

To our knowledge no data are available on the plasma protein binding of the catecholamines and their O-methylated metabolites. Our results show that the binding is of a low order i. e., 14 to 23 % in physiological concentration. This seems rational since adrenaline at least is released into the bloodstream and acts on the peripheral organs. The low binding of the catecholamines also rules out the possibility that part of the circulatory response to intravenously injected amphetamine could be due to the displacement of endogenous amines from a plasma protein pool.

Acknowledgements

This study was supported by grants from the Swedish Medical Research Council (14X-2211), Swedish Delegation for Applied Medical Defense Research No. 278 the C. B. Nathhorst Foundation.

REFERENCES

Axelrod, J. Studies on sympathomimetic amines. II. The biotransformation and physiological disposition of D-amphetamine, D-p-hydroxyamphetamine and D-methamphetamine. *J. Pharm. Exp. Ther.* 1954, 110, 315–326.

Bixler, H. J. Membrane ultrafiltration. The diafiltration technique and its application to microsolute exchange and binding phenomena. *Anal. Biochem.* 1968, 26, 151–173.

Borgå, O., D. Azarnoff & F. Sjöqvist: Species differences in the plasma protein binding of desipramine. *J. Pharm. Pharmac.* 1968, 20, 571.

Borgå, O., D. Azarnoff, G. Plym Forshell & F. Sjöqvist: Plasma protein binding of tricyclic antidepressants in man. *Biochem. Pharmacol.* 1969, 18, 2135–2143.

Connell P H. Amphetamine psychosis. Maudsley Monographs No. 5 Oxford University Press, London (1958)

Garattini, S. & E. Costa (Eds.): International symposium on amphetamines and related compounds. Raven Press, New York (1969).

Kalant, C. J The amphetamines; toxicity and addition. University of Toronto Press (1966)

Schach von Wittenau, M. & R. Yeary: The excretion and distribution in body fluids of tetracyclines after i.v. administration to dogs. *J Pharmacol. Exp Ther* 1963, 140 258–266.

Sjöqvist, F & M. Tottie (Eds.): Abuse of central stimulants. Almqvist & Wiksell, Stockholm (1969).

Acta pharmacol. et toxicol. 1970, 28 215–224

From the Department of Pharmacology
University of Copenhagen, Denmark

In Vitro Studies on the Uptake of ^{14}C Labelled Decamethonium and Hexamethonium in Mouse Liver

By

C. Broen Christensen

(Received December 16, 1969)

Abstract. The uptake process of ^{14}C-decamethonium in liver was studied by measuring its uptake in slices incubated in Krebs-Ringer bicarbonate medium (37° pH 7.4) and for comparison the ^{14}C-hexamethonium uptake was measured. At 2 µM the slice-to-medium (S/M) ratio for decamethonium was about 3 after 1 hour and continued to increase. The hexamethonium uptake after 1 hour was only ½ of the decamethonium uptake. Slices from female and immature male mice accumulated decamethonium to the same extent as slices from male mice, whereas rat liver slices were unable to concentrate decamethonium. In an atmosphere of nitrogen and in the presence of 1 µM 2,4-dinitrophenol, iodoacetic acid or cyanide the decamethonium uptake was significantly reduced. With increasing concentration of decamethonium the 1-hour S/M ratio decreased towards a constant value (1.5). Thus the uptake could be divided into a passive and saturable process. The latter had a maximum capacity of 81 µM kg^{-1} h^{-1} and a half saturation concentration of 54 µM. With 2 µM the decamethonium S/M ratio was reduced to about 50 % in the presence of 50 µM d-tubocurarine. The inhibitory effect of 22 µM d-tubocurarine decreased with increasing decamethonium concentration in accordance with competitive antagonism.

Key-words: Decamethonium compounds – hexamethonium compounds – liver

Several publications have appeared in recent years reporting the accumulation of methonium compounds in isolated tissue. Thus McIsaac (1965) found that hexamethonium is concentrated in slices of cat kidney incubated in a medium containing ^{14}C-hexamethonium. Using a similar method it has been demonstrated that decamethonium is concentrated in the isolated rat dia phragm (Taylor *et al.* 1965) and in slices from the cerebral cortex of the rat (Taylor *et al.* 1968).

We have demonstrated in our laboratory that decamethonium is concen trated in slices from the mouse liver by a process which is partly energy

dependent, and that d-tubocurarine can inhibit the uptake to a considerable extent (BROEN CHRISTENSEN 1967a & 1967b) In addition we have presented chromatographic evidence of the accumulation of decamethonium as the unchanged compound in mouse liver (BROEN CHRISTENSEN & HOLM 1969).

The object of the present work has been to study the uptake process of decamethonium in liver tissue more thoroughly and to attempt to describe this quantitatively with the intention of comparing it with the uptake in other tissues and in other animal species. In addition we have compared the uptake of decamethonium and hexamethonium in the liver

Materials and methods

The following isotope labelled compounds have been used. ^{14}C-*decamethonium* dibromide (Radiochemical Centre, Amersham, England) with a specific activity of 20.9 mci/mM, ^{14}C-hexamethonium chloride (New England Nuclear Corp., U. S. A.) with a specific activity of 1.55 mci/mM and ^{14}C-sucrose (Radiochemical Centre, Amersham, England) with a specific activity of 10.6 mci/mM

The following non-labelled compounds have been used. decamethonium dibromide ("Syncurine Burroughs Wellcome & Co.), d-tubocurarine chloride (Abbott), g-strophantin (Ph. Nord. 1963) and 2.4 (α) dinitrophenol, iodoacetic acid and potassium cyanide (Merck).

Preparation of liver slices and incubation procedure.

Albino male mice of between 25 and 27 g have been used unless otherwise stated.

The animals were decapitated and bled, after which the liver was rapidly removed *and prepared. A portion of liver was removed and sliced by hand with a razor* blade. Four slices from each liver of between 0.3 and 0.5 mm in thickness were placed in a tube containing 20 ml of Krebs-Ringer bicarbonate medium.

Th medium had the following composition (meq./l)· N 144 K 4.7 Ca^{++} 5.0· Mg^{++} 2.4 Cl^- 130· HCO_3 25 H_2PO_4 1.2 and in addition it also contained 2 g/l of glucose.

The slices were preincubated for 10 minutes at 37° During this preincubation and also later the medium was gassed with a mixture of oxygen-carbon dioxide (95/5 v/v %) and the pH of the medium became 7.4 In the experiments using anaerobic conditions nitrogen-carbon dioxide mixture (95/5 /v %) was used producing a pH of a similar value namely 7.4 A mixing of the medium was ensured by shaking the tubes (60 oscillations/min.). The same amount of ^{14}C-decamethonium was added to each tube together with varying amounts of non-labelled decamethonium. In the experiments in which metabolic inhibitor was used this was added to the medium 10 minutes before the decamethonium, but in the experiments in which d-tubocurarine was used this was added to the medium just before the decamethonium. At the end of the incubation period the slices were separated from the medium by filtration on cotton. The wet weight of the slices from each liver was 200–300 mg.

Estimation of radioactivity in tissue and medium.

A method previously described (*BROEN CHRISTENSEN 1965) for measuring* ^{14}C-decamethonium in tissue samples and plasma was used for the analysis of the radioactivity

in the slices and the medium. The method is based on hydrolysis of the tissue with NaOH and protein precipitation with $Zn(OH)_2$. The measurements were carried out on Packard Tri-carb fluid scintillation spectrometer model 314 EX. Control with internal standard showed that ^{14}C-activity was registered with the same efficiency in the tissue samples as in the samples of the medium.

Calculation of the slice-to-medium (S/M) concentration ratio.

Slice-to-medium concentration ratio was calculated as $\frac{\text{counts per minute/g "slices"}}{\text{counts per minute/ml medium}}$ inasmuch as the wet weight was used as reference at the end of the incubation period. The dry-matter content of the slices was determined by drying at 105° for 24 hours. After incubation for 1 hour the dry-matter percentage was 20.4 ± 0.4 (Mean ± S. E. M., N = 6). Before incubation the dry-matter percentage was 31.0 ± 0.8 (Mean ± S. E. M. N = 6).

The S/M ratio expresses the volume of medium (ml), which contains the same amount of decamethonium as found in 1 g of tissue. As the bulk of the tissue is small in relation to the volume of the medium, the decamethonium concentration in the medium will be practically constant within the experimental period (30–120 minutes).

Results

Decamethonium uptake as a function of time.

A rapid accumulation of decamethonium in the tissue occurs when slices of liver are incubated in a medium containing decamethonium (2 μM). The concentration in the slices is already approximately twice the concentration in the medium after 30 minutes (fig. 1) and the ratio increases continuously within an incubation period of 2 hours. With incubation under anaerobic conditions the uptake of decamethonium is considerably less and does not increase to any great extent on incubation for more than 1 hour.

In an attempt to estimate the size of the extracellular space in the liver slices we have determined the slice-to-medium concentration ratio of ^{14}C sucrose. After incubation for 1 hour the ratio is 0.54 ± 0.02 (Mean ± S. E. M., N = 6). However the ratio increases with longer incubation and after 2 hours reaches a value of 0.73 ± 0.04 (Mean ± S. E. M., N = 6).

Decamethonium uptake in adult female and immature male mice.

The above results have been obtained from liver slices from male mice weighing 25–27 g. For comparison the concentration ratio for decamethonium in slices from female mice of the same weight category are shown in table 1. It can be seen that there is no significant sex difference with regard to the decamethonium uptake. In addition it can also be seen from the table that the age of the male animals has no influence on the uptake, inasmuch as the same concentration ratio is found with slices from 3 weeks old male mice as in the control group.

dependent, and that d-tubocurarine can inhibit the uptake to a considerable extent (BROEN CHRISTENSEN 1967a & 1967b). In addition we have presented chromatographic evidence of the accumulation of decamethonium as the unchanged compound in mouse liver (BROEN CHRISTENSEN & HOLM 1969).

The object of the present work has been to study the uptake process of decamethonium in liver tissue more thoroughly and to attempt to describe this quantitatively with the intention of comparing it with the uptake in other tissues and in other animal species. In addition we have compared the uptake of decamethonium and hexamethonium in the liver

Materials and methods

The following isotope labelled compounds have been used. ^{14}C-decamethonium dibromide (Radiochemical Centre, Amersham, England) with a specific activity of 20.9 mci/mM, ^{14}C-hexamethonium chloride (New England Nuclear Corp., U.S.A.) with a specific activity of 1.55 mci/mM and ^{14}C-sucrose (Radiochemical Centre, Amersham, England) with a specific activity of 10.6 mci/mM.

The following non-labelled compounds have been used. decamethonium dibromide ("Syncurine" Burroughs Wellcome & Co.), d-tubocurarine chloride (Abbott), g-strophantin (Ph. Nord. 1963) and 2.4 (α) dinitrophenol, iodoacetic acid and potassium cyanide (Merck).

Preparation of liver slices and incubation procedure

Albino male mice of between 25 and 27 g have been used unless otherwise stated.

The animals were decapitated and bled, after which the liver was rapidly removed and prepared. A portion of liver was removed and sliced by hand with a razor blade. Four slices from each liver of between 0.3 and 0.5 mm in thickness were placed in a tube containing 20 ml of Krebs-Ringer bicarbonate medium.

The medium had the following composition (meq./l): N^{+} 144 K^{+} 4.7 Ca^{++} 5.0 Mg^{++} 2.4 Cl^{-} 130 HCO_3^{-} 25 $H_2PO_4^{-}$ 1.2 and in addition it also contained 2 g/l of glucose.

The slices were preincubated for 10 minutes at 37°. During this preincubation and also later the medium was gassed with a mixture of oxygen-carbon dioxide (95/5 v/v %) and the pH of the medium became 7.4. In the experiments using anaerobic conditions a nitrogen-carbon dioxide mixture (95/5 v/v %) was used producing a pH of a similar value namely 7.4. A mixing of the medium was ensured by shaking the tubes (60 oscillations/min.). The same amount of ^{14}C-decamethonium was added to each tube together with varying amounts of non-labelled decamethonium. In the experiments in which a metabolic inhibitor was used this was added to the medium 10 minutes before the decamethonium, but in the experiments in which d-tubocurarine was used this was added to the medium just before the decamethonium. At the end of the incubation period the slices were separated from the medium by filtration on cotton. The wet weight of the slices from each liver was 200–300 mg.

Estimation of radioactivity in tissue and medium.

A method previously described (BROEN CHRISTENSEN 1965) for measuring ^{14}C-decamethonium in tissue samples and plasma was used for the analysis of the radioactivity

Table 2.

Decamethonium uptake in slices of rat liver after 60 and 120 minutes incubation. The uptake is expressed as slice-to-medium (S/M) concentration ratio. The decamethonium concentration in the medium was 2 μM. Results are given as the mean of values in 6 experiments ± S. E. M

Incubation period (min.)	60	120
S/M ratio	0.83 ± 0.06	0.99 ± 0.04

By comparing the results shown in table 1 and fig. 1 it can be seen that liver tissue from this animal species accumulates decamethonium to a slight extent only inasmuch as the slice-to-medium ratio does not become greater than 1 after incubation for 2 hours. Albino male rats of 150–180 g were used for the experiments. The thickness of the slices and their weight were as described in the experiments with mouse liver

Hexamethonium uptake as a function of time.

Fig. 2 shows the results of incubation experiments with mouse liver slices in a medium containing hexamethonium (2 μM). The S/M ratio only reaches 1 after incubation for 1 hour which corresponds to ⅓ of the decamethonium uptake under identical conditions. In the hexamethonium experiments an almost steady-state was obtained with incubation for 2 hours, which is far from the case with decamethonium.

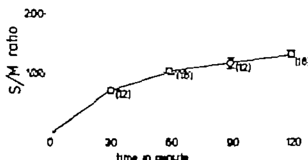


Fig. 2. Slice-to-medium (S/M) concentration ratio of hexamethonium as a function of time. The hexamethonium concentration in medium was 2 μM Each point represents the mean value of experiments in an atmosphere of oxygen. Figures in brackets indicate number of experiments and the vertical bars S. E. M.

Table 3

Effect of various inhibitors on the uptake *of decamethonium in liver slices from mice.* The uptake is expressed as slice-to-medium (S/M) concentration ratio after 1 hour incubation. The decamethonium concentration in medium was 2 µM. Results are given as mean of values from 6 experiments ± S. E. M. (In control group 36 experiments).

Inhibitor	Concentration (M)	S/M ratio
2,4-dinitrophenol	10^{-6}	1.28 ± 0.03
	10^{-4}	2.26 ± 0.04
Iodoacetic acid	10^{-6}	1.37 ± 0.02
	10^{-4}	2.56 ± 0.18
Potassium cyanide	10^{-6}	1.91 ± 0.15
Ouabain	10^{-5}	2.90 ± 0.12
None		2.96 ± 0.07

The effect of metabolic inhibitors on decamethonium uptake.

Various metabolic inhibitors reduce the uptake of decamethonium (table 3). The greatest effect is obtained with dinitrophenol, which in a concentration of 1 µM inhibits the uptake to the same extent as under anaerobic conditions. Iodoacetic acid *1* µM and potassium cyanide *1* µM *similarly* inhibit the uptake significantly whereas ouabain 10 µM has no definite effect.

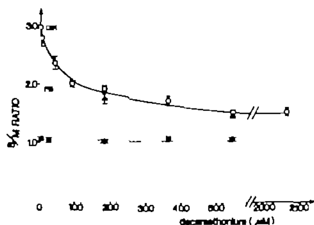


Fig. 3. Slice-to-medium (S/M) concentration ratio of decamethonium after 1 hour incubation as a function of the decamethonium concentration *in the medium.* Open circles and unbroken line represent S/M ratio in oxygen atmosphere. Closed circles and dotted line represent S/M ratio in nitrogen atmosphere. The three triangles indicate results from experiments in an atmosphere of oxygen in the presence of 22 µM d-tubocurarine. Figures in brackets indicate number of experiments. When not stated points represent mean values from 5–6 experiments. The vertical bars indicate S. E. M.

Decamethonium uptake as a function of the decamethonium concentration in the medium.

The results in fig. 3 were obtained by varying the concentration of decamethonium in the medium. During aerobic conditions the slice-to-medium concentration ratio is seen to fall with increasing decamethonium concentration towards a constant value around 1.5 This value is significantly higher than the concentration ratio under anaerobic conditions, which remains constant around 1 with all concentrations of decamethonium.

Based on the course of the curve one would assume that the decamethonium uptake under aerobic conditions consists of two processes, i. e. a passive process with a constant S/M ratio around 1.5 and a saturable process. The concentration ratio for the saturable process (Sa/M) can be calculated as the difference between the concentration ratio for the whole process and the concentration ratio for the passive process. A value of 1.53 has been used for this which is the mean figure for S/M ratio at 540 and 2060 μM. In fig. 4 the reciprocal value of Sa/M has been plotted against the decamethonium concentration in the medium (M) in the same way as in DIXON & WEBB's (1964) method for the quantitative evaluation of enzymatic reactions. As the relation, as far as can be seen, is linear it is possible to calculate the maximum capacity of the process as the reciprocal value of the slope of the line and to read the half saturation concentration as the negative value of the point at which the line intersects the abscissa. Thus calculated the maximum capacity is found to be 81 μM kg^{-1} h^{-1} and the half saturation concentration to be 54 μM.

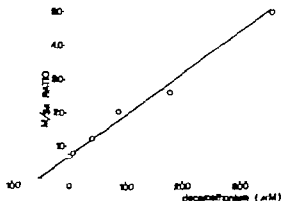


Fig. 4 Relation between the reciprocal of saturable decamethonium uptake (M/Sa ratio) and the external concentration of decamethonium (μM). Data from fig. 3 corrected for passive diffusion (Sa/M = S/M − 1.53 for details, see legend of fig. 3 and text).

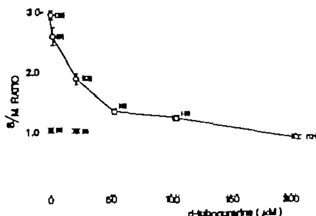


Fig. 5 The inhibitory effect of d-tubocurarine on the decamethonium uptake (S/M ratio) after 1 hour incubation. The decamethonium concentration in the medium was 2 μM. Points in open circles represent mean values of experiments in an atmosphere of oxygen. Points in closed circles represent mean values of experiments in an atmosphere of nitrogen. Figures in brackets indicate number of experiments and the vertical bars S. E. M. The S/M ratio in the presence of d-tubocurarine, 204 μM is significantly lower than the S/M ratio in an atmosphere of nitrogen. ($0.02 > P > 0.01$).

The effects of d-tubocurarine on decamethonium uptake

In fig. 5 the decamethonium S/M ratio has been drawn as the function of the d-tubocurarine concentration. It can be seen that a part of the uptake is very sensitive to low concentrations of d-tubocurarine. With 50 μM d-tubocurarine the total uptake is reduced by somewhat more than 50 / With higher concentrations the uptake is inhibited even more, but the sensitivity of the process to the inhibitor becomes considerably lower It should be noted that with a d-tubocurarine concentration of 204 μM an uptake is found that is significantly lower than in the experiments under anaerobic conditions without the presence of inhibitors. Some results are also shown in fig. 3 where the S/M ratio is depicted as a function of the decamethonium concentration of incubation in the presence of 22 μM d-tubocurarine in the medium. It can be seen that the inhibitory effect of d-tubocurarine decreases with increasing decamethonium concentrations and that at 540 μM there is no significant difference in the results either with or without the inhibitor

Discussion

The time course of decamethonium uptake in liver slices from mice corresponds fairly well with the course of decamethonium uptake in slices of rat cerebral cortex (TAYLOR *et al.* 1968). In these studies where the external

decamethonium concentration was 1 μM, a steady state was obtained in the distribution of decamethonium after incubation for 7 hours with a S/M ratio around 11. This high ratio together with the very slow approach to the steady state must mean that the release of decamethonium from the tissue does not play any important role in short incubation experiments. A steady state was already obtained in our experiments under anaerobic conditions after incubation for approximately 1 hour with a S/M ratio of slightly more than 1 (fig.1) Even though this ratio is low as compared with the S/M ratio under aerobic conditions, it is however greater than the S/M ratio for sucrose, which suggests that a part of the decamethonium taken up is bound to tissue structures.

As shown the uptake of decamethonium can be divided into 2 components: one that is directly proportional to the concentration in the medium and another component, which follows saturation kinetics. A similar kinetics have been described for the accumulation of ^{14}C-choline (SCHUBERTH *et al.* 1966) and ^{14}C-acetylcholine (SCHUBERTH & SUNDWALL 1967) in slices of mice cerebral cortex. The capacity of the saturable component is however considerably greater for choline and acetylcholine in nerve tissue than for decamethonium in liver tissue Studies in our laboratory on the accumulation of decamethonium in kidney tissue from mice (HOLM & BROEN CHRISTENSEN 1969) also support the assumption that there is no qualitative difference in the uptake of depolarizing compounds in excitable and non-excitable cells. The possibility should be mentioned that decamethonium uses a phytiological transport system for choline The presence of such a system in human erythrocytes has recently been demonstrated by MARTIN (1968). MARTIN (1969) later showed that decamethonium and other neuromuscular blocking compounds are potent inhibitors of choline transport. However his experiments showed that decamethonium does not penetrate into human erythrocytes.

Our study on the effect of d-tubocurarine suggests that the inhibition is of a competitive nature and that it is the saturable component of the uptake that is sensitive to the curare, but our data do not allow of conclusions being drawn on these points.

In studies on the uptake of carbacholine in slices from the cerebral cortex of rats CREESE & TAYLOR (1967) were successful in identifying the curare sensitive component of the uptake as the saturable component.

The low uptake of hexamethonium in liver tissue is in agreement with our *in vivo* studies on mice (BROEN CHRISTENSEN & HOLM 1969), which demonstrated a considerable difference between the distribution of hexamethonium and decamethonium. There is also a considerable difference in the fate of these two methonium compounds in the cat, but in contrast to the conditions in the mouse, in the cat kidney there is a considerable accumulation of hexamethonium and no accumulation of decamethonium (McISAAC 1969).

The great difference between the uptake of decamethonium and hexamethonium in mouse liver possibly reflects different affinities for a common carrier. Experiments with subcellular structures of liver cells are in progress in an attempt to localize the binding sites for methonium compounds.

Acknowledgements

The Packard Tri-Carb Scintillation spectrometer of the department was purchased with a grant given by the Danish State Research Foundation (A 5/65). Mrs. Karin Jørgen-Jensen gave technical assistance.

REFERENCES

Broen Christensen, Chr. Distribution and biliary excretion of decamethonium in doubly nephrectomized rabbits. *Acta pharmacol. et toxicol.* 1965, 23 275–286.

Broen Christensen, Chr. Decamethonium uptake by slices of mouse liver. *Biochem. Pharmac.* 1967a, 16 2221–2222.

Broen Christensen, Chr. Decamethonium uptake in the liver of intact mice and by slices of mouse liver. *Acta pharmacol. et toxicol.* 1967b, 25 Suppl. 4, 46.

Broen Christensen, Chr. & J. Holm. The distribution and urinary excretion of decamethonium and hexamethonium following intravenous injection into mice. *Acta pharmacol. et toxicol.* 1969 27 17–26.

Creese, R. & D. B. Taylor. Entry of labelled carbachol in brain slices of the rat and the action of d-tubocurarine and strychnine. *J. Pharmacol. Exp. Ther.* 1967 157 406–419.

Dixon, M. & E. C. Webb. *Enzymes* 1964 2nd ed., pp. 325–331. London, Longmans, Green & Co. Ltd.

Holm, J. & Chr. Broen Christensen. Decamethonium uptake by slices of mouse kidney and the inhibitory effect of d-tubocurarine. *Acta physiol. scand.* 1969 77 Suppl. 330, 69.

Martin, K. Concentrative accumulation of choline by human erythrocytes. *J. gen. Physiol.* 1968 51, 497–516.

Martin, K. Effects of quaternary ammonium compounds on choline transport in red cells. *B. J. Pharmac.* 1969 36, 458–469.

McIsaac, R. J. The uptake of hexamethonium-C14 by kidney slices. *J. Pharmacol. Exp. Ther.* 1965 150 92–98.

McIsaac, R. J. The binding of organic bases to kidney cortex slices. *J. Pharmacol. Exp. Ther.* 1969 168, 6–12.

Schubert, J. A. Sundwall, B. Sörbo & J.-O. Lindell. Uptake of choline by mouse brain slices. *J. Neurochem.* 1966, 13, 347–352.

Schubert, J. & A. Sundwall. Effects of some drugs on the uptake of acetylcholine in cortex slices of mouse brain. *J. Neurochem.* 1967 14 807–813.

Taylor, D. B., R. Creese, O. A. Nedergaard & R. Case. Labelled depolarizing drugs in normal and denervated muscle. *Nature* 1965, 208 901–902.

Taylor, D. B., R. Creese & T.-C. Lu. The mode of action of morphine on the uptake of carbachol and decamethonium by slices of rat cerebral cortex. *J. Pharmacol. Exp. Ther.* 1968, 165 310–319.

Acta pharmacol. et toxicol. 1970, 28, 225–232.

From the Department of Pharmacology, Institute of Pharmacy
University of Oslo, Norway

An Assay Method for Trasylol® Based on the In Vitro Inhibition of Human Plasma Kallikrein

By

Gudrun Briseid

(Received November 28, 1969)

Abstract. Plasma kallikrein prepared by activation of citrated plasma with acetone (16.7 % v/v) for 17 hours at room temperature (20–24°) was preincubated with trasylol for 10 minutes (37°). Kinin release was obtained by incubation for 30 minutes (37°) with citrated human plasma in which the kininase had been eliminated with EDTA-2Na (4 mg per ml) for 30 minutes (37°), and in which the acetone-susceptible kallikrein inhibitors had been depressed immediately before by the addition of acetone (12.5 % v/v). The reaction was stopped by heating for 30 minutes (100°) after which the released kinin was assayed on the isolated rat uterus. Ten different trasylol samples were estimated against a standard trasylol in (2 + 2) assays with a dose ratio 1 : 0.5 and with 6 series of 4 doses. The average departure from the true trasylol content was 5.4 % (range − 8.8 to + 11.1 %) and the mean systematical deviation was + 0.4 %. The fiducial limits ranged from 95–105 % to 89–112 % (P = 0.05), and s/b was 0.035 (range 0.024 to 0.053).

Key-words: Kallikrein-trypsin inactivator – kallikrein.

Substances with inhibitory effects on various proteolytic enzymes have been isolated from bovine organs. KRAUT, FREY & WERLE (1930) detected a kallikrein inhibitor in the parotid gland, the spleen, the liver and the lymph nodes. KUNITZ & NORTHROP (1936) reported the isolation of a trypsin inhibitor from the pancreas, and ASTRUP (1952) discovered an inhibitor for plasmin in lung extracts. Several similarities in the properties of the inhibitors suggested that they were identical (WERLE, MAIER & RINGELMANN 1952, MARX, CLEMENTE, WERLE & APPEL 1959, TRAUTSCHOLD & WERLE 1961, WERLE 1964, KRAUT, BHARGAVA, SCHULTZ & ZIMMERMANN 1963, KRAUT & BHARGAVA 1963, KRAUT & BHARGAVA 1964). According to VOGEL, TRAUTSCHOLD & WERLE (1968) all the polyvalent bovine proteinase inhibitors are identical.

The commercial preparation trasylol® contains a polypeptide obtained from bovine lung tissue and also from bovine parotid gland, which produces inhibitory effects on trypsin, plasmin, chymotrypsin and various kallikreins.

A number of methods have been suggested for the quantitative determination of trasylol. The preparation was originally assayed by its inhibitory effect on the fall in blood pressure produced by kallikreins in the dog (FREY KRAUT & WERLE 1950) The potency was expressed in kallikrein inhibitor units (KIU) (WERLE & KAUFMANN-BOETSCH 1960) Assay methods based on the inhibition of plasmin have been described by MARX, CLEMENTE, WERLE & APPEL (1959) KALLER (1963) AUHAGEN (1964) BELLER, EPSTEIN & KALLER (1966). TRAUTSCHOLD & WERLE (1961) developed a photometric method in which trasylol inhibited the hydrolysis of benzoylarginine ethyl ester caused by kallikrein. The method was modified in order to increase its sensitivity (WERLE & TRAUTSCHOLD 1961)

In connection with the discussion on the possible effects of trasylol in therapy considerable interest has been focused on the significance of the inhibition of the plasma kinin system. Accordingly it should be appropriate to base an assay method on the inhibitory effect on human plasma kallikrein. The term "plasma kallikrein includes all kininogenases activated by acetone. The method described in the present paper was based on the inhibition by trasylol of the release of kinin in human citrated plasma caused by plasma kallikrein (BRISEID, ARNTZEN & DYRUD 1968 BRISEID, DYRUD & ARNTZEN 1968)

Technique

1. *Human plasma kininogen preparation* was prepared and stored as described by BRISEID, DYRUD & ARNTZEN (1968). In the present work the pooled plasma obtained from 4 men in the age range of 25 to 31 years was used for the preparation.

2. *A etone-activated human plasma kallikrein* was prepared and stored according to BRISEID, DYRUD & ARNTZEN (1968)

3. *Standard and test kallikrein inhibitor* Trasylol® in ampoules of 100,000 kallikrein inactivator units (KIU) in 10 ml sterile, isotonic solution, Bayer A. G Leverkusen, Germany

4. *Rat uterus* The kinin determinations were carried out on the isolated rat uterus as previously described (BJELTVEDT & BRISEID 1967).

5. *Method.* To 2 samples of acetone-activated human plasma kallikrein was added standard trasylol in dose ratio of 1 0.5 (80 and 40 inhibitor units per 0 045 ml enzyme preparation). To 2 other samples test trasylol was added in amounts which according to a preliminary assay corresponded to the standard doses. Each of the samples was diluted with saline to 0.19 ml per 0.045 ml enzyme preparation and preincubated for 10 minutes at 37° This was followed by incubation for kinin release

in 1 ml preparation of human plasma kininogen. Just before the addition of trasylol-inhibited plasma kallikrein, acetone-susceptible inhibitors in the plasma kininogen preparation were depressed by the addition of acetone corresponding to a final concentration of 12.5 % (/v) in the samples to be incubated (BRISEID, ARNTZEN & DYRUD 1968). After incubation at 37° for 30 minutes 0.6 ml samples were withdrawn and the reaction stopped by dilution with 5 ml of boiling saline and heating for 30 minutes in a boiling water bath. The cooled samples were diluted to 10 ml with saline and kept at 4 for assay within 24 hours.

The assay was carried out as (2 + 2) assay with 6 series of 4 randomized doses. In order to obtain the same concentration of plasma all the tests were equally diluted with de Jalon's solution. The individual contractions were measured to the nearest mm and the statistical procedure described in the Nordic Pharmacopoeia was used for the calculation of the results.

6. *Reagents. Bradykinin* (synthetic) in ampoules of 100 µg per ml, Sandoz, A. G Basel, Switzerland. *Ethylenediami tetra-acetic acid disodium salt (EDTA 2N) Titriplex III* Merck, A. G Darmstadt, Germany *Acetone p a.*, Merck, A. G Darmstadt, Germany

Comments on the Technique

1. *Human plasma kininogen preparation.* According to MARGOLIS & BISHOP (1963) the addition of an excess amount of plasma kallikrein to human plasma caused a release of kinin corresponding to about ⅓ of total kininogen present, while complete exhaustion of the kininogen required the presence of acetone in a sufficiently high concentration to suppress the kallikrein inhibitors. JACOBSEN (1966a & b) and JACOBSEN & KRIZ (1967) also found that plasma kallikrein released approximately ⅓ of the total kininogen and he was able to isolate kininogen 1 corresponding to this fraction and in addition also a kininogen 2. According to BRISEID, DYRUD & ARNTZEN (1968) the rate of release of kinin from the remaining kininogen fraction, which reacts with plasma kallikrein only in the presence of acetone, is so slow that it has no practical significance in the present work (30 minutes). Thus, the basis for the proposed method is the trasylol-induced inhibition of kinin release from the minor kininogen fraction.

2. *Acetone-activated human plasma kallikrein.* The enzyme preparation used seems to contain more than one kininogenase. In the absence of cetone only a minor fraction of the kininogen of the plasma substrate preparation was activated, and according to BRISEID, DYRUD & ARNTZEN (1968) and BRISEID, DYRUD & ØIE (1970) this partial release is probably due to the rapid inactivation by inhibitors in the substrate of the kallikrein fraction, acting on the main kininogen. The enzyme reacting with the minor kininogen was considered far more stable. The acetone-susceptible kallikrein inhibitors in the plasma kallikrein preparation are inactivated during the 17-hour incubation period with 16.7 % (/v) acetone.

3. *Method. Plasma kallikrein concentration effect curves.* Fig. 1 curve A shows a typical kinin release curve. For the plasma kininogen preparation used, th kinin released by an excess amount of plasma kallikrein (1 ml enzyme preparation per ml plasma kininogen preparation) was 1 7 µg per ml plasma calcul ted as bradykinin, while the total kininogen determined by the acetone method (BRISEID, DYRUD & RØVIK 1967) was 4 6 µg. Curves established with other enzyme preparations and other plasma kininogen preparations showed similar courses.

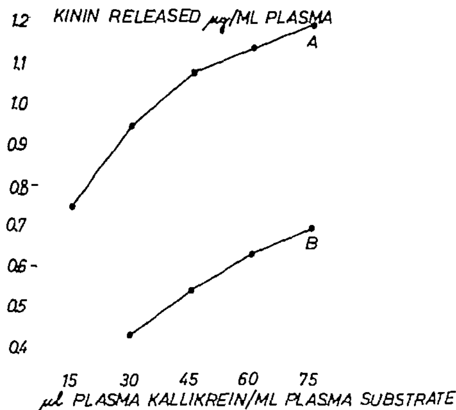


Fig. 1. *Human plasma kallikrein dose effect curves.*
Plasma substrate: Human citrated plasma, stabilized with EDTA 2Na, 4 mg/ml plasma substrate.
Plasma kallikrein. Human citrated plasma, activated with acetone, 16.7 % (/v), for 17 hours at room temperature before addition of EDTA-2Na.
Incubation period. 15 minutes.
Curve A No trasylol added
Curve B Trasylol 57 KIU/dose of plasma kallikrein and ml plasma substrate.
Kinin released calculated as µg bradykinin/ml plasma.

Fig. 1, curv B shows a concentration effect curve obtained with plasma kallikrein inhibited by trasylol (57 KIU per 0.030 to 0.075 ml enzyme per ml plasma kininogen preparation). The experiment was carried out simultaneously with the experiment shown in fig. 1, curve A, and the same enzyme and the same substrate were used. The curves were almost parallel and the inhibition about 50 %

The actual concentrations of plasma kininogen preparation per 100 ml de Jalon's solution ranged from 0.02 to 0.11 ml (average 0.06 ml)

Trasyl l concentration ffect curve Bradykinin was used as the standard substance during the development of the method, for example in the establishment of dose effect curves with plasma kallikrein (fig. 1) with trasylol-inhibited plasma kallikrein (fig. 2), and for the time effect curve with trasylol-inhibited plasma kallikrein (fig 3).

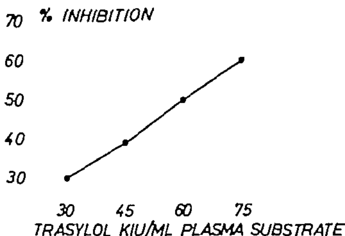


Fig. 2. *Trasylol dose inhibition curve*
Plasma substrate and plasma kallikrein, see fig. 1.
Plasma kallikrein: 0.045 ml/trasylol dose/ml plasma substrate.
Incubation period. 15 minutes.
Kinin released by 0.045 ml plasma kallikrein/ml plasma substrate: 1.1 μg calculated as bradykinin.

Fig. 2 shows the straight curve for trasylol inhibition in the range 30 to 75 KIU per 0.045 ml kallikrein per ml plasma kininogen preparation. The same enzyme and the same kininogen preparations were used as in the experiments shown in fig. 1. When trasylol was not present, 0.045 ml plasma kallikrein caused a release of 1.1 μg kinin calculated as bradykinin per ml plasma kininogen preparation, or 65 % of the maximum release value.

A possible effect of trasylol on the bradykinin stimulated rat uterus was examined for concentrations of the inhibitor up to 0.70 KIU per ml de Jalon's solution. No effect was observed. The actual concentrations of trasylol per ml de Jalon's solution ranged from 0.04 to 0.10 KIU (average 0.06 KIU) when only the higher doses of the (2 + 2) assays shown in table 1 were considered.

Th inhibition by acetone of kallikrein inhibitors in the plasma kininogen preparation. Acetone inhibits not only inhibitors of plasma kallikrein, but to some extent also kallikrein itself. Small alterations in the acetone concentration resulted in considerabl deviations in the amounts of kinin released, and the greatest accuracy was accordingly necessary in the pipetting.

Preincubation period. Experiments were carried out to estimate the preincubation time necessary for enzyme and trasylol to obtain maximum inhibition with the experimental conditions chosen, i.e. 30 and 75 KIU per 0.045 ml enzyme. At the lowest inhibitor concentration used the inhibition was complete after 5 minutes at 37° while 10 minutes were required for the highest amount of inhibitor used. Accordingly 10-minute preincubation period was chosen for the method. Fig. 3 shows t curves for kinin release with trasylol-inhibited plasma kallikrein.

Table I

Accuracy and precision of the method.
Different trasylol solutions assayed on uteri from different rats.
s/b Ratio of standard deviation to the slope of the regression line.

Assay	Trasylol units/ml Theoretical	Observed in (2 + 2) assay	Actual error as % of true potency	s/b	Fiducial limits % (P = 0.05)
1	740	800	+ 8.1	0.029	94–106
2	860	910	+ 5.8	0.032	93–107
3	1150	1130	− 1.7	0.045	91 110
4	650	660	+ 1.5	0.035	93–107
5	920	890	− 3.3	0.024	95–105
6	1200	1160	− 3.3	0.038	92 109
7	990	1100	+ 11.1	0.053	89–112
8	680	620	− 8.8	0.028	94–106
9	870	800	− 8.0	0.030	93–107
10	790	810	+ 2.5	0.032	94–107

Incubation period. In preliminary experiments 15-minute incubation period was used. This procedure caused a relatively low per cent kinin release which resulted in a relatively high concentrations of plasma in de Jalon's solution. With plasma concentrations ranging from 0.2 to 0.4 ml per 100 ml test solution, irregularities of the uterine preparations were observed rather often and several had to be rejected. When the incubation period was increased to 30 minutes, the concentration of plasma decreased correspondingly and the assay difficulties were largely eliminated.

Results

To test the accuracy and precision of the suggested method 10 different trasylol solutions were assayed. The strengths of the solutions were unknown to the observer Uterine preparations from different rats were used. The results are shown in table 1 The statistical procedure described in the Nordic Pharmacopoeia was used in dealing with the results. Table 1 shows that the precision was satisfactory The range of fiducial limits (P = 0 05) varied from 95–105 / to 89–112 / and the values for the ratio of standard deviation to the slope of the regression line (s/b) were from 0 024 to 0 053 (mean value 0 035) The standard and test curves were parallel except in 2 experiments which were 0.05 > P > 0 01 The accuracy of the method was satisfactory The average departure from the theoretical content of trasylol was 5 4 % (range −8 8 to +11.1 /) and the mean systematical deviation was +0 4 %

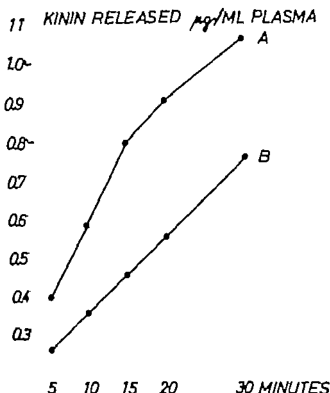


Fig. 3. *Time effect curves for trasylol inhibited plasma kallikrein.*
Plasma substrate and plasma kallikrein, see fig. 1.
Curve A. Trasylol 30 KIU/0.045 ml plasma kallikrein/ml plasma substrate.
Curve B. Trasylol 75 KIU/0.045 ml plasma kallikrein/ml plasma substrate.
Kinin released calculated as μg bradykinin/ml plasma.

REFERENCES

Astrup, T. On lung tissue as a proteolytic inhibitor. *Acta physiol. scand.* 1952, 26, 243–251.

Anhagen, J. Verteilung und Elimination von Trasylol bei der Maus. *Dissertation* Düsseldorf 1964.

Beller, F. K., D. Epstein & H. Kaller: Distribution, half-life time and placental transfer of the protease inhibitor Trasylol. *Thrombos. Diathes. Haemorrh.* 1966, 16, 302–310.

Bleitvedt, G. & K. Briseid: An assay method for padutin® (hog pancreas kallikrein) based on the *in vitro* release of kinin in human plasma. *Acta pharmacol. et toxicol.* 1967, 25, 190–196.

Briseid, K., F. C. Arntzen & O. K. Dyrud. Studies on the release of kinin in human plasma. *Acta pharmacol. et toxicol.* 1968, 26, 402–412.

Briseid, K., O. K. Dyrud & F. C. Arntzen: Determination of prekallikrein in human plasma. *Acta pharmacol. et toxicol.* 1968, 26, 395–401.

Briseid, K., O. K. Dyrud & S. F. Rimvik: *In vitro* estimation of the rate of release of kinin in human plasma. *Acta pharmacol. et toxicol.* 1967, 25, 201–213.

Briseid, K., O. K. Dyrud & S. Öie: Determination of factors of the kinin system in rat plasma. *Acta pharmacol. et toxicol.* 1970, 28, 124–137.

Frey, E. K., H. Kraut & E. Werle: *Kallikrein (Padutin)*. Ferdinand Enke Verlag, Stuttgart 1950.

Jacobsen, S.: Separation of two different substrates for plasma kinin-forming enzymes. *Nature* 1966a, 210, 98–99.

Jacobsen, S.: Substrates for plasma kinin-forming enzymes in human, dog and rabbit plasmas. *Brit. J. Pharmacol.* 1966b, 26, 403–411.

Jacobsen, S. & M. Kriz: Some data on two purified kininogens from human plasma. *Brit. J. Pharmacol.* 1967, 29, 25–36.

Kaller, H.: Tierexperimentelle Untersuchungen über Wirkungsdauer und Elimination von Trasylol®. *Arch. exp. Path. Pharmak.* 1963, 246, 92–93.

Kraut, H., N. Bhargava, F. Schultz & H. Zimmermann: Versuche zur Isolierung des Kallikrein-Inaktivators, Kristallisation und Aminosäure Zusammensetzung, Vergleich mit dem Trypsin-Inhibitor von Kunitz und Northrop. *Z. physiol. Chem.* 1963, 334, 230–235.

Kraut, H. & N. Bhargava: Das Verhalten des Kallikrein-Inaktivators aus Rinderparotis gegenüber Fermenten, II. *Z. physiol. Chem.* 1963, 334, 236–242.

Kraut, H. & N. Bhargava: Versuche zur Isolierung des Kallikrein-Inaktivators. V. Isolierung eines Kallikrein-Inaktivators aus Rinderlunge und seine Identifizierung mit dem Inaktivator aus Rinderparotis. *Z. physiol. Chem.* 1964, 338, 231–237.

Kraut, H., E. K. Frey & E. Werle: Über die Inaktivierung des Kallikreins. *Z. physiol. Chem.* 1930, 192, 1–21.

Kunitz, M. & J. H. Northrop: Isolation from beef pancreas of crystalline trypsinogen, trypsin and an inhibitor trypsin compound. *J. gen. Physiol.* 1936, 19, 991–1007.

Margolis, J. & E. A. Bishop: Studies on plasma kinins. I. The composition of kininogen complex. *Aust. J. exp. Biol. med. Sci.* 1963, 41, 293–306.

Marx, R., P. Clemente, E. Werle & W. Appel: Zum Problem eines Antidots in der internen Thrombotherapie mit Fibrinolytika. *Blut* 1959, 5, 367–375.

Trautschold, I. & E. Werle: Spektrophotometrische Bestimmung des Kallikreins und seiner Inaktivatoren. *Z. physiol. Chem.* 1961, 325, 48–59.

Vogel, R., I. Trautschold & E. Werle: *Natural proteinase inhibitors.* Academic Press, New York & London 1968.

Werle, E.: Über einen Hemmkörper für Kallikrein und Trypsin in der Rinderlunge. *Z. physiol. Chem.* 1964, 338, 228–230.

Werle, E. & B. Kaufmann-Boetsch: Über esteratische Wirkungen von Kallikrein und Trypsin und ihre Hemmung durch Kallikrein- und Trypsininhibitoren. *Z. physiol. Chem.* 1960, 319, 52–63.

Werle, E., L. Maier & E. Ringelmann: Hemmung von Proteinasen durch Kallikrein-Inaktivatoren. *Naturwiss.* 1952, 39, 328.

Werle, E. & I. Trautschold: Bestimmung des Kallikrein-Inaktivators "Trasylol" in Blut und in Organen und seine Verteilung nach Injektionen. *Münch. med. Wschr.* 1961, 103, 773–775.

Acta pharmacol. et toxicol. 1970 28 233–240

From the Department of Pharmacology Royal Veterinary College, Stockholm, Sweden

Lethal Brain Concentrations of Nicotine in Mice of Different Ages

By

Torbjörn Stålhandske and Premysl Slanina

(Received February 27 1970)

Abstract: Albino mice aged 3 days, 12 days and 35 days, were injected intraperitoneally with lethal doses of ^{14}C-nicotine. At death the concentrations of nicotine in the brain and blood were measured. Significant age dependent differences between lethal brain and blood concentrations and latent period before death occurred were found. The brains f mice aged 12 days were found t have the lowest and the brains of mice aged 35 days the highest nicotine concentration at death. The lethal concentration of nicotine in the blood was highest in mice aged 3 days and lowest in mice aged 12 days. Brain/blood ratios increased with age. The latent period before death occurred was shortest in mice aged 12 days and longest in mice aged 3 days. The significance of these conditions for age dependent differences in tolerance to nicotine is discussed.

Key-words: Nicotine – brain.

Age-dependent changes in drug susceptibility have been demonstrated in man and other mammalian species. Frequently the premature infant or neonatal animal is intolerant to drug doses calculated on a weight basis which can be tolerated by the adult. Increased sensitivity has been reported for a variety of compounds: CNS depressants" analgesics, anticholinergic compounds and antibiotics (for review see DONE 1964)

The central nervous system "stimulants offer an interesting contrast to this pattern of increased sensitivity in the young: generally young animals are less sensitive to the toxic effects of CNS stimulants than are adults (YEARY *et al* 1966).

This phenomenon has been demonstrated for strychnine, pentetrazole and nicethamide (SETNIKAR & MAGISTRETTI 1964 PYLKKÖ & WOODBURY 1961 FERNGREN 1965). A similar phenomenon has also been found in the case of nicotine. STÅLHANDSKE *et al* (1969) reported that mice aged 3 days could

tolerate doses of nicotine as high as adults. However in contrast they showed that mice aged 12 days had developed a very high sensitivity to the toxic effects of nicotine. The same authors suggested that the subsequent decrease in sensitivity observed in older mice varied directly with the development of enzymatic detoxication in the liver However in mice aged 3 days other factors must be involved as these mice tolerated doses as high as in adults despite a much lower capacity of detoxication.

The central nervous system in which nicotine is largely taken up (SCHMITERLÖW *et al.* 1967) is prominently involved in the overall pharmacology of nicotine (for review see SILVETTE *et al.* 1962) so that the lethal effects of nicotine in mice are very likely due to its central effects (ACETO *et al.* 1969).

The aim of the present study was to investigate whether the differences in susceptibility to nicotine are related to a change in the sensitivity of the CNS with age, i. e. if different brain concentrations of nicotine are required to cause death.

Material and methods

Experimental animals.

White mice of the NMRI strain aged 3 12 and 35 days were used. The approximate ages of the experimental animals purchased on a weight basis were determined according to an age and weight curve previously constructed. In the group of 35 days old mice, the sexes were equally represented. In each age group 12–17 animals were used. Mice aged 3 and 12 days were kept together with their mothers until they were used.

Compounds.

Nicotine-methyl-^{14}C was synthesized according to the method of MCKENNIS *et al.* (1962) as described by HANSSON & SCHMITERLÖW (1962) The purity was checked by thin-layer chromatography (STÅLHANDSKE 1970) and the specific activity was 28 µci/mg Nicotine bitartrate was used as a source of non-labelled nicotine, 1 g corresponding to 0.329 g of the pure base.

Animal experiments.

Mice were injected intraperitoneally with ^{14}C-nicotine dissolved in distilled water In order to obtain the necessary amount of nicotine in the injected solution non-labelled nicotine bitartrate was added to the radioactive base. The amount of nicotine injected was calculated as the pure base.

Thus different solutions were made up one with a specific activity of 2.6 µci/mg, which was used for mice aged 3 and 35 days, and another with a specific activity of 11.3 µci/mg, which was used for 12 days old mice.

The mice aged 3 and 35 days were given 23 mg/kg and the 12 days old mice 5.4 mg/kg of nicotine. The doses corresponded to the 2-fold LD50 dose according to STÅLHANDSKE *et al.* (1969.) The total volume injected for 20 g mice was 0.1 ml and this was reduced according to the weight of the animals. For injection into the youngest animals a Hamilton 100 µl microsyringe was used and the site of injection was pinched to avoid leakage. In 12 and 35 days old mice death was always preceded

by convulsions. The pattern of the convulsions corresponded to that described in the previous investigations (excitation, tremor jerking movements, clonic and tonic convulsions and death) (YAMAMOTO *et al.* 1966). Mice aged 12 and 35 days were considered t be dead when the body relaxed after the tonic convulsions. In control ex periments no breathing occurred and no animals survived after the occurrence of tonic convulsions.

The character of the seizures differed in the 3 days old animals. Trembling and hyperkinesia occurred and this was followed by loss of body posture and finally by slow and intense paddling movements of the legs. The respiratory rate became slow with periods of apnoea and the animals progressively became more cyanotic and pale. When breathing stopped for more than 15 seconds a foot was pinched with a pair of tweezers. This sometimes induced further breathing. When no more breathing could be induced the animal was considered to be dead.

When the experimental animals were dead the head was immediately cut off and the blood collected in 3 ml of heparinized ice cold 0.2 M phosphate buffer and weighed. In 3 days old animals the blood collected from 2 to 3 animals was pooled. The brains were removed and weighed. The blood and brain tissues were immediately frozen and kept at − 20 until the nicotine was extracted.

Determination of nicotine.

The brains were homogenized in 0.2 M phosphate buffer pH 7.4. From the homogenates and blood samples ^{14}C-nicotine was extracted according to HUCKER *et al.* (1960). The amount of radioactive nicotine thus extracted was determined in Packard Tri-Carb liquid scintillation counter after addition of ethanol and 1 per cent PPO in toluene. The extraction was checked by thin-layer chromatography

In all determinations the radioactivity was counted for a period sufficient to yield at least 1,000 counts. Values in counts per minute were converted to values in micrograms per sample, based on the specific activity of nicotine used and on the absolut counting efficiencies determined with standards of ^{14}C-toluene.

Statistical evaluation f data.

Student's "t distribution was used as a test of the null hypothesis. The level of significance used was $P <$ than 0.05

Results

The highest average lethal *brain* concentration of nicotine (8.35 μg/g) was observed in 35 days old mice. In the 12 days old mice the brain concentration on an average was 75 per cent and in 3 days old mice 42 per cent lower than in 35 days old mice (table 1). The differences in brain concentrations between the different ages were significant.

However the highest average lethal nicotine concentration in the *blood* (8.75 μg/g) was observed in 3 days old mice. In 12 days old mice the concentration in the blood was on an average 76 per cent and in 35 days old mice 49 per cent lower than in 3 days old mice. The differences were significant.

Table I

Nicotine in brains of mice at death.

Age (days)	Mean body wt. (g)	Mean brain wt (g)	Acute toxicity LD50 [mg/kg body wt. and 95 % confidence limits*]	Lethal concentrations of nicotine (µg/g wet wt) ± S. E. M.		Brain/Blood ± S. E. M.	Time of death (sec.) ± S. E. M.
				Brain	Blood		
35	19.5	0.375	11.5 [10.0–12.9]	1) 8.35 ± 1.02 (16)	1) 4.47 ± 0.47 (16)	1) 1.91 ± 0.16 (16)	1) 157 ± 17 (16)
12	5.4	0.342	2.7 [1.4–5.2]	2) 2.10 ± 0.15 (12)	2) 2.11 ± 0.33 (9)	2) 1.08 ± 0.14 (9)	2) 98 ± 8 (11)
3	2.7	0.132	11.2 [9.7–12.8]	3) 4.87 ± 0.66 (17)	3) 8.75 ± 1.11 (8)	3) 0.58 ± 0.10 (8)	3) 375 ± 25 (14)
				1 2 P < 0.001 2–3 P < 0.05 1 3 P < 0.05	1 2 P < 0.001 2–3 P < 0.001 1 3 P < 0.005	1 2 P < 0.001 2–3 P < 0.02 1 3 P < 0.001	1 2 P < 0.005 2–3 P < 0.005 1–3 P < 0.02

Figures in brackets indicate number of experiments.

*) Determined by STÅLHANDSKE *et al.* 1969.

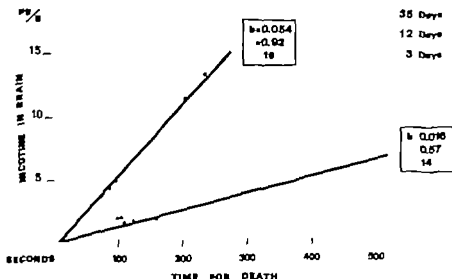


Fig. 1. Correlations between lethal concentrations of nicotine in the brain and time of death in 3 12 and 35 days old mice. The correlation was significant for 35 days ($P < 0.001$) and 3 days ($P < 0.05$) old mice. The slopes b = 0.054, and b = 0.01 differed significantly ($P < 0.05$).

Significant differences between the brain to blood ratios of nicotine concentrations were also observed. The highest ratio 1 91 was observed in the oldest mice and decreased with age to 1 08 (12 days) and 0.57 (3 days)

On an average the *time of survival* after the nicotine injection also differed significantly with age and was longest in the youngest mice (375 seconds) and shortest in 12 days old mice (98 seconds) (table 1).

A significant correlation between the lethal nicotine concentrations in the brain and the time of survival was found in 3 and 35 days old mice. However no such correlation was found in 12 days old mice.

The slopes of the two regression lines (fig. 1) were also found to be significantly different.

Discussion

On the assumption that in mice the cause of death with nicotine is of central origin (ACETO *et al.* 1969) the concentration of nicotine in the brain at death may be considered as a measure of the sensitivity of the brain to the lethal effects of nicotine. The significant differences between lethal brain

concentrations of nicotine obtained with the doses used in mice of different ages, thus indicate the presence of an age dependent change in the sensitivity of the brain to nicotine.

The changes in the lethal brain concentrations with age correlate fairly well with the change in the LD50 values.

It would be attractive, particularly in the case of 12 and 35 days old mice where ratios between LD50 values and lethal brain concentrations were similar to attribute the differences in LD50 values to a change in brain sensitivity to nicotine However other mechanisms must also be taken into consideration.

In addition to the fact that LD50 determinations are inexact and crude criteria for the determination of toxicity the LD50 value is the result of a number of possible age dependent variable conditions. Each of these conditions may change with age causing an increase or decrease of the dose necessary for a lethal effect.

Some examples of probable age dependent changeable conditions are absorption, metabolism (detoxication), distribution to and affinity for pharmacologically active and silent receptors. Age dependent changes of drug distribution within the CNS are known to occur (for review see ROTH & BARLOW 1965) and it is known that the nicotine detoxicating capacity of the liver gradually increases with age (STÅLHANDSKE *et al.* 1969) When analyzing known age dependent changes of nicotine metabolism and distribution the following discussion may be relevant.

From the age of 12 to 35 days there is an increase in the average lethal nicotine concentration in the brain and an increase in the detoxicating capacity of the liver These changes acting synergistically progressively increase the lethal dose of nicotine. However they are antagonized by an increasing capacity of the brain to accumulate nicotine, as is indicated by the increased brain/blood ratio seen in 35 days old mice.

Nevertheless the result is an increase in the LD50 from 2.7 to 11.5 mg/kg indicating that conditions which produce an increase in LD50 outweigh the condition responsible for a decrease of the lethal dose

From the age of 3 days to 12 days there is a decrease in the average lethal nicotine concentration in the brain and an increased capacity of the brain to accumulate nicotine as indicated by the increased brain/blood ratio

These conditions change synergistically leading to a decrease of the lethal dose of nicotine. However the capacity of the liver to detoxify nicotine increases progressively with age (STÅLHANDSKE *et al.* 1969) and thus gradually increases the lethal dose of nicotine. In these circumstances in which the LD50 is decreased from 11.2 to 2.7 mg/kg the factors which operate toward a decrease of the lethal dose outweigh the factor producing an increase of the lethal dose.

Although the LD50 values are similar in 3 and 35 days old mice the average lethal nicotine concentration in the brains was lower in 3 days old mice. However according to the brain/blood ratios the affinity of nicotine for brain tissue was lower in 3 days old mice. The uptake was also definitely slower in 3 days than in 35 days old mice, since the slopes of the regression lines illustrating the uptake of nicotine in brain with time, differed significantly. The slow uptake at this age may be one of the causes of the long latent period before death occurs.

Although a central component is thought to be of primary importance in causing nicotine death in adult mice this may be secondary in the newborn. PYLKKÖ & WOODBURY (1961) who found a similar sensitivity trend with age for strychnine and brucine in rats attributed the high tolerance to a lack of functional synapses in CNS of newborn animals.

This may also be the reason for the low sensitivity of nicotine and the less dramatic toxic syndrome seen in newborn mice (STÅLHANDSKE *et al.* 1969). In contrast to young mice the primary cause of death in the newborn may be a progressively increasing peripheral curare-like paralysis of the respiratory muscles (for review see SILVETTE *et al.* 1962). This and the high resistance of newborn for hypoxic states (for review see JILEK *et al.* 1964) may also be the explanation for the increase in the latent period before death occurs.

The high sensitivity of the brain for nicotine at the age of 12 days may be attributed to the fact that the development of the brain proceeds rostrally with phylogenetically primitive hindbrain structures maturing earlier than the younger forebrain systems (RICHTER 1967). Neonatal animals are believed to pass through a phase when the major excitatory system in the hindbrain reticular formation is functional but in which the forebrain inhibitory mechanisms are not fully developed (CAMPBELL *et al.* 1969). During this phase the life preserving functions of the central nervous system may be more easily damaged by nicotine.

Acknowledgements

This work was supported by grants to Professor Carl G. Schmiterlöw from the Swedish Tobacco Company, the American Medical Association Education and Research Foundation, and Knut och Alice Wallenbergs Stiftelse (for general equipment for isotope research work).

The skilful technical assistance of Mrs. A. Österblom and Mrs. B. Hagtorn is gratefully acknowledged.

REFERENCES

Aceto, M. D., H. C. Bentley & J. R. Dembinsky: Effect of ganglion blocking agents on nicotine extensor convulsions and lethality in mice. *Brit. J. Pharmacol.* 37. 104–111.

Campbell, B. A., L. D Lytle & H. C. Fibiger Ontogeny of adrenergic arousal and cholinergic inhibitory mechanisms in the rat. *Science*. 1969 166 637–638.

Done, A. K. Developmental pharmacology *Clin. Pharmac Ther* 1964, 5, 432–479

Ferngren, H. Pentetrazol induced seizures and their antagonism by barbiturates in newborn and young mice. *Acta pharmacol. et toxicol.* 1965, 23, 27–35

Hansson, E. & C. G. Schmiterlöw Physiological disposition and fate of 14C-labelled nicotine in mice and rats. *J Pharmac. exp Ther* 1962, 137 91–102.

Hucker H. B., J R. Gillette & B. B. Brodie: Enzymatic pathways for the formation of cotinine, a major metabolite of nicotine, in rabbit liver *J Pharmoc. exp Ther* 1960 129 94–100.

Jilek, L., J Fischer L. Krulich & S. Trojan: The reaction of the brain to stagnant hypoxia and anoxia during ontogeny In. *Progress in brain research*. vol. 9 (Ed. Himwich and Himwich). Elsevier publishing company Amsterdam 1964 pp. 113–131.

McKennis, Jr H., L. B Turnbull, S. L. Schwartz, E. Tamaki & E. R. Bowman: Demethylation in the metabolism of ( )nicotine. *J biol. Chem*. 1962, 237 541 546.

Pylkkö, O O. & P M. Woodbury The effect of maturation on chemically-induced seizures in rats. *J Pharmac. exp. Ther* 1961, 131, 185–190.

Richter D.. Enzymic activity in the nervous system. In. *Regional development of the brain in early life*. (Ed. A. Minkowski). Blackwell Scientific Publications, Oxford and Edinburgh 1967 pp. 137–156.

Roth, L. J & C. F Barlow Autoradiography of drugs in the brain In. *Isotopes in experimental pharmacology* (Ed. L. J Roth). The University of Chicago Press, Chicago and London 1965 5, 49–62.

Schmiterlöw C. G E. Hansson, G Andersson, L.-E. Appelgren & P C. Hoffmann. Distribution of nicotine in the central nervous system. *Ann. N Y Acad. Sci.* 1967 142, 2–14

Setnikar I. & M. J Magistretti. The toxicity of central nervous system stimulants in rats of different ages. *Proceedings of the European Society for th Study of Drug T icity* Excerpta Medica Foundation. 1964 4 132–139

Silvette, H., E. C. Hoff, P S. Larson & H. B Haag: The actions of nicotine on central nervous system functions. *Pharmacol. Rev* 1962, 14 137 173.

Stålhandske, T P Slanina, H. Tjälve, E. Hansson & C. G. Schmiterlow Metabolism in vitro of 14C-nicotine in livers of foetal, newborn and young mice. *Acta pharmacol. et t xicol.* 1969 27 363–380

Stålhandske, T The metabolism of nicotine and cotinine by a mouse liver preparation. *Acta physiol. scand* 1970, 78, 236–248.

Yamamoto, I., K. Otori & R. Inoki. Pharmacological studies on antagonists against nicotine-induced convulsions and death. *Jap. J Pharmac*. 1966, 16 402–415

Yeary R. A., R. A. Benish & M. Finkelstein. Acute toxicity of drugs in newborn animals. *J Pediat* 1966, 69 663–667

Acta pharmacol. et toxicol. 1970 28 241–248

From H. Lundbeck & Co. A/S,
Departments of Synthetic Chemistry (1) and Pharmacology (2),
Copenhagen – Valby Denmark

Further Pharmacological Studies of Bicyclic Thymoleptics

By

P V Petersen N. Lassen[1] T A[illegible][1]
L Møller Nielsen[2] M. Nymark[2] V Pedersen[2]
and K. F Franck[2]

(Received October 16, 1969)

Abstract: The reserpine antagonistic and noradrenaline (NA) potentiating activities of 19 phthalans and 18 thiophthalane derivatives have been compared with those of 7 known tricyclic thymoleptics. Thirty minutes after an intraperitoneal injection of the test compound to mice, reserpine (2 mg/kg intraperitoneally) was given and one hour later the degree of ptosis was scored. The activity of each compound was expressed as the dose (mg/kg intraperitoneally) that reduced the ptotic score to half that of control mice (ED50). The NA potentiating effect was estimated in the pithed rat preparation. A small submaximally active dose (0.05 µg/kg intravenously) of NA was given as standard. After the intravenous injection of the test drug, the augmentation of the NA pressor response was calculated as a percentage of the initial value. The ED50 was read from a dose-effect curve. It was found that 3,3-dimethyl-1-(3-aminopropyl) substituted phthalanes and thiophthalanes with a phenyl group or certain other electro-negative, noncyclic groups in position 1, were the most potent reserpine antagonists and NA potentiators. The most interesting compounds were Lu 3-010 (3,3-dimethyl 1 (3-methylaminopropyl)-1-phenyl-phthalane), Lu 4-012 (3,3-dimethyl-1-(3-methylaminopropyl)-1-cyano-phthalane) and Lu 5-003 (3,3-dimethyl-1-(3-methylaminopropyl)-1-phenyl-thiophthalane), which showed marked reserpine antagonism and NA potentiation, but which were without any neuroleptic properties and had very weak anticholinergic effects

Key words Antidepressive agents – psychopharmacology amitriptyline – nortriptyline – imipramine – desipramine.

In a previous publication (PETERSEN *et al.* 1966) the reserpine antagonistic and noradrenaline (NA) potentiating activities of various bicyclic compounds were compared with those of known tricyclic thymoleptics. It was found that 1-phenyl-1-(3-aminopropyl)-3,3-dimethyl substituted phthalane derivatives were particularly potent.

Following this further derivatives were synthesized in order to study the structure activity relationship with regard to thymoleptic properties. In this study 1-(3-aminopropyl)-phthalanes with various substituents in the ring

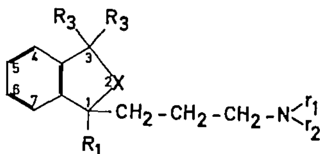


Fig. 1 General structure of the bicyclic compounds examined.

structure were examined and compared with Lu 3-010 (3,3-dimethyl-1-(3-methylaminopropyl)-1-phenyl-phthalane) and the clinically known tricyclic thymoleptics. In addition a number of analogous thiophthalane derivatives were included in the study The general structure of the compounds studied is given in fig. 1.

Table 1

Structure of phthalanes.

Serial no.	Code no	X	R_1	R_3	R_3	r_1	r_2	Salt	Melting point C
1	Lu 3-010	O	C_6H_5	CH_3	CH_3	CH_3	H	HCl	190–91
2	Lu 4-004	O	C_6H_5	CH_3	CH_3	H	H	oxalate	198–99
3	Lu 3-021	O	p-F-C_6H_4	CH_3	CH_3	CH_3	CH_3	HCl	150–52
4	Lu 3-073	O	p-F-C_6H_4	CH_3	CH_3	CH_3	H	HCl	205–06
5	Lu 3-102	O	$COCH_3$	CH_3	CH_3	CH_3	CH_3	HCl	154–55
6	Lu 4-003	O	$COCH_3$	CH_3	CH_3	CH_3	H	HCl	159–60
7	Lu 3-098	O	COC_2H_5	CH_3	CH_3	CH_3	CH_3	HCl	191–92
8	Lu 5-006	O O	$COCH(CH_3)_2$	CH_3	CH_3	CH_3	CH_3	HCl	187–89
9	Lu 3-105	O	COC_6H_5	CH_3	CH_3	CH_3	CH_3	HCl	184–85
10	Lu 4-002	O	COOH	CH_3	CH_3	CH_3	CH_3	HCl	188–89
11	Lu 4-006	O	$COOCH_3$	CH_3	CH_3	CH_3	CH_3	HCl	169–70
12	Lu 4-001	O	$COOC_2H_5$	CH_3	CH_3	CH_3	CH_3	HCl	154–55
13	Lu 3-109	O	$CONH_2$	CH_3	CH_3	CH_3	CH_3	HCl	226–27
14	Lu 4-073	O	$CONHC_6H_5$	CH_3	CH_3	CH_3	CH_3	oxalate	136–38
15	Lu 3-092	O	CN	CH_3	CH_3	CH_3	CH_3	HCl	165–67
16	Lu 4-012	O	CN	CH_3	CH_3	CH_3	H	HCl	180–82
17	Lu 4-050	O	CN	CH_3	CH_3	H	H	HCl	212–14
18	Lu 4-010	O	OH	H	H	CH_3	CH_3	oxalate	104–06
19	Lu 6-041	O	C_6H_5	CF_3	CF_3	CH_3	CH_3	HCl	167–69

Table 2

Structure of thiophthalanes.

Serial no.	Code no.	X	R_1	R_2	R_3	r_1	r
20	Ln 4-074	S	C_6H_5	CH_3	CH_3	CH_3	[illegible]
21	Ln 5-003	S	C_6H_5	CH_3	CH_3	CH_3	H
22	Ln 5-060	S	C_6H_5	CH_3	CH_3	H	[illegible]
23	Ln 4-071	S	C_6H_5	CH_3	H	CH_3	[illegible]
24	Ln 5-001	S	C_6H_5	CH_3	H	CH_3	[illegible]
25	Ln 4-064	S	C_6H_5	H	H	CH_3	[illegible]
26	Ln 4-070	S	C_6H_5	H	H	CH_3	[illegible]
27	Ln 5-099	S	C_6H_5	CH_3	CH_3	C_6H_5	[illegible]
28	Ln 5-055	S	$COCH_3$	CH_3	CH_3	CH_3	[illegible]
29	Ln 5-045	S	COOH	CH_3	CH_3	CH_3	[illegible]
30	Ln 5-046	S	$COOCH_3$	CH_3	CH_3	CH_3	[illegible]
31	Ln 5-042	S	$COOC_2H_5$	CH_3	CH_3	CH_3	[illegible]
32	Ln 5-040	S	$CONH_2$	CH_3	CH_3	CH_3	[illegible]
33	Ln 5-044	S	$CONHC_6H_5$	CH_3	CH_3	CH_3	[illegible]
34	Ln 5-052*	S=O	C_6H_5	CH_3	CH_3	CH_3	H
35	Ln 5-054	S=O	C_6H_5	CH_3	CH_3	CH_3	H
36	Ln 5-071	SO_2	C_6H_5	CH_3	CH_3	CH_3	[illegible]
37	Ln 5-069	SO_2	C_6H_5	CH_3	CH_3	CH_3	H

The two isomers were completely separated by fractional cryst

Materials and Methods

Chemical.

The compounds studied are given in table 1 (phthalanes) an
The compounds were prepared as the most convenient wa
in tables 1 and 2. Results given in the following table
weighed.

Pharmacological.

Acute toxicity was determined by intravenous inje
weighing 18–22 g, kept in single cages at about 23° The L
dose levels with 5 mice per group were determined by the
(1944).

Reserpine antagonism.

1) A preliminary estimation of the anti-reserpine a
test compound intraperitoneally at the dose levels of
each of 3 male mice. The animals were then observ
tion or stimulation) for one hour Then stand
given intravenously Observation of the occurrence
or absence of the typical reserpine syndrome wa
untreated control groups were included in each
reserpine after the first hour The degree of antag

was roughly estimated on a scale ranging from 0 to +++ The observer was unaware of the identity of the experimental groups until after the experiment. This test was used only as a quick guide to the compounds that might be most suitable for the more detailed study

2) Prevention of reserpine-induced ptosis. Unfasted male mice (NMRI) in the weight range of 18–25 g were grouped 5 to a cage and treated with the test drug. An untreated group served as control. The injections of test compounds were done by an investigator different from the observer the latter not being informed about the identity of the groups until after scoring. After half an hour had elapsed, all the animals were given reserpine (serpasil® 2.0 mg/kg) and returned to their original cages. All injections ere given intraperitoneally in a volume of 0.1 ml/10 g of bodyweight. One hour after the administration of the challenging dose of reserpine the cages were tilted up and down a few times, and 30 seconds later the animals were scored for the degree of ptosis.

The scoring system adopted by RUBIN *et al.* (1957) was used 4 = complete, 3 = ¾ 2 = ½ 1 = ¼ closure of the eyelids. Normal opening was scored zero. If different degrees of ptosis in the two eyes of an animal were observed, the average score was used. The activity of each test drug was expressed as the dose (mg/kg) that reduced the ptotic score to half that of the control mice (ED50). For some of the more potent compounds higher doses (32 and 64 mg/kg) were given to see whether the degree of antagonism against the reserpine syndrome would decrease (reversal).

Table 3

Activity of phthalanes.

Serial no.	Code no.	LD 50 mg/kg i.	Reserpine Antagonism: Prelim.	Ptosis ED 50 mg/kg i. p.	Reversal mg/kg i. p.	NA Potent. ED 50 mcg/kg i. v.
1	Lu 3-010	71	+++	0.4	> 64	10
2	Lu 4-004	97	++(+)	6		100
3	Lu 3-021	74	++(+)	0.6		900
4	Lu 3-073	103	+++	∼ 1.5		440
5	Lu 3-102	47	+++	∼ 8		>> 1000
6	Lu 4-003	43	+++	6		760
7	Lu 3-098	45	++(+)	∼ 8		> 1000
8	Lu 5-006	48	++	> 8		> 1000
9	Lu 3-105	41	+++	∼ 1.5		500
10	Lu 4-002	> 200	+	> 8		
11	Lu 4-006	48	++(+)	2		300
12	Lu 4-001	61	+++	2	> 64	160
13	Lu 3-109	110	++(+)	> 8		∼ 1000
14	Lu 4-073	25	+++	2	> 64	∼ 1000
15	Lu 3-097	44	+++	∼ 1	> 64	340
16	Lu 4-012	59	+++	0.25	> 64	45
17	Lu 4-050	71	+	7.5		> 1000
18	Lu 4-010		+	> 8		
19	Lu 6-041	40	+	20		> 1000

Table 4

Activity of thiophthalanes and reference substances.

Serial no.	Code no.	LD 50 mg/kg i. v.	Reserpine Antagonism: Prelim.	Ptosis ED 50 mg/kg i. p.	Reversal mg/kg i. p	NA Potent. ED 50 mcg/kg i. v
20	Lu 4-074	49	+++	1	> 64	350
21	Lu 5-003	67	+++	~ 1	> 64	60
22	Lu 5-060	66	+(+)	16		> 1000
23	Lu 4-071	48	+++	8		> 1000
24	Lu 5-001	60	+++	5		350
25	Lu 4-064	48	++	12		> 1000
26	Lu 4-070	60	+++	6		400
27	Lu 5-099	34	(+)	>> 8		>> 1000
28	Lu 5-055	17	-	>> 8		>> 1000
29	Lu 5-045	> 200	0	>> 8		>> 1000
30	Lu 5-046	23	-	~ 12		800
31	Lu 5-042	21		~ 2		700
32	Lu 5-040	98	+++	3.5	> 64	> 1000
33	Lu 5-044	34	++	6	> 64	900
34	Lu 5-052	106	(+)	> 8		600
35	Lu 5-054	123	(+)	>> 8		> 1000
36	Lu 5-071	90	0	>> 8		
37	Lu 5-069	125	+(+)	42		
38	Amitriptyline	38	+(+)	14	32	~ 4000
39	Nortriptyline	39	+(+)	6	32	2100
40	Imipramine	40	+	3	64	350
41	Desipramine	49	++(+)	1	32	70
42	Melitracen	52	++(+)	4	> 64	2500
43	Litracen	38	+++	2	> 64	780
44	Protriptyline	49	+++	0.7	64	25

50 mg/kg i. p. lethal.

The noradrenaline (NA) potentiating effect was estimated in the pithed rat preparation of SHIPLEY & TILDEN (1947) as described previously (MØLLER NIELSEN & NEUHOLD 1959). A small submaximally active dose, e. 0.05 µg/kg intravenously of NA giving pressor response of 10–20 mm Hg was used as standard. After the intravenous injection of the test drug, the augmentation of the NA response was calculated as a percentage of the initial value. When 1 mg/kg of the test drug yielded less than 50 % potentiation and the anti-reserpine effect was found to be weak, usually no further doses were tested, and the result (tables 3 and 4) was given as > 1000 (weak effect) or ≪ 1000 (extremely weak effect). In other experiments at least 3 dose levels (= 2) were tested, and a dose-effect curve was drawn on semi-logarithmic paper from which the ED50 was read. A few drugs were tested at dose of 8 mg/kg to see whether a maximal potentiation was still present or whether reversal had occurred.

Results and Discussion

In tables 1 to 4 the compounds are identified by code number or generic name for the reference substances. In order to facilitate reference to any one compound they have also been assigned a serial number (column 1)

Among the phthalanes (tables 1 and 3) the most active compounds were Lu 3-010 and Lu 4-012 (serial Nos. 1 and 16) This means that the most favourable substitution in position 1 is either a phenyl- or a CN-group. Substitution in position 1 by para-F-C_6H_4 (Lu 3-021 and Lu 3-073 serial Nos. 3 and 4), $COOCH_3$ and $COOC_2H_5$ (Lu 4-006 and Lu 4-001 serial Nos. 11 and 12) still retained considerable activity while other substitutions resulted in marked reduction of activity

From our previous work with this type of compound it was known that dimethyl substitution in position 3 was essential for potent reserpine antagonism and NA potentiation.

Replacement of the methyl groups with CF_3 (Lu 6-041 No 19) resulted in considerable loss of activity It is of interest that the primary amine Lu 4-004 (No 2) which has been found to be a metabolite of Lu 3-010 (PLYM FORSHELL *et al* 1968) still has some anti-reserpine effect and is quite active in potentiating NA. Lu 4-050 (No 17) which may be assumed to be a metabolite of Lu 4-012 (No 16) does not seem to have retained much of the activity of the parent compound.

Lu 4-012 (No 16) besides being a very potent reserpine antagonist and NA potentiator also seemed to possess a certain central stimulating effect causing hyperactivity and a tendency to stereotype head movements at a dose level of 50 mg/kg. This has never been observed with Lu 3-010 (No 1).

In the thiophthalane series (tables 2 and 4) the most active compound was Lu 5-003 (No.21) the direct analogue to Lu 3-010 (No. 1) which had an activity very close to that of desipramine (No. 41).

The primary amine Lu 5-060 (No 22) and the two sulphoxides Lu 5-052 and Lu 5-054 (Nos. 34 and 35) were included since they have been identified as metabolites of Lu 5-003 (FREDRICSON OVERØ *et al* 1970) They all appear to be weak reserpine antagonists. It is interesting that Lu 5-052 (No 34) still retained some NA potentiating effect.

The present results seem to indicate that the structural characteristics, shown to be essential for the powerful reserpine antagonism and NA potentiation of the phthalanes (PETERSEN *et al.* 1966), also seem to be essential for the effect of the thiophthalanes. 1) substitution by a phenyl group or certain other electro-negative, non-cyclic groups in position 1 2) substitution by two CH_3-groups in position 3 3) a three carbon side chain 4) a secondary amine group in the side chain. The thiophthalanes, however appear to be slightly less active than the corresponding phthalane derivatives.

Some of the compounds included have been investigated for reversal of reserpine antagonism, and it may be seen from tables 3 and 4 that none of the bicyclic compounds showed reversal of antagonism. In the pithed rat preparation intravenous doses as high as 8 mg/kg of Lu 3-010 and Lu 5-003 still showed maximal potentiation of the NA pressor response. With the same doses of protriptyline and desipramine the potentiation was markedly reduced.

The previously described bicyclic compounds were found to possess a very weak anticholinergic activity while the known tricyclic thymoleptics are quite strong anticholinergics (PETERSEN *et al.* 1966) On the conventional guinea-pig ileum preparation Lu 5-003 was also very weak, being about 630 times less active than atropine. Lu 4-012 was completely devoid of anticholinergic activity

CARLSSON *et al.* (1969) have studied the effects of some of the bicyclic compounds on the membrane pump mechanism of central and peripheral monoamine neurones. Lu 3-010 was found to be extremely potent in inhibiting the amine uptake at the cell membrane of peripheral NA neurones. Lu 5-003 was somewhat weaker On central NA neurones, however the relative potencies tended to be the reverse. Thus, the ability of Lu 5-003 to block amine uptake centrally was distinctly superior to that of Lu 3-010 and even desipramine. In view of these results Lu 5-003 seems to be one of the most potent inhibitors of the membrane pump mechanism of the central NA neurones.

REFERENCES

Carlsson, A., K. Fuxe, B. Hamberger & T Malmfors: Effect of a new series of bicyclic compounds with potential thymoleptic properties on the reserpine-resistant uptake mechanism of central and peripheral monoamine neurones in vivo and in vitro. *Brit. J Pharmacol.* 1969 36, 18–28.

Fredricson Overø, K., A. Jørgensen & V Hansen. Metabolism, distribution and excretion of the thiophthalane Lu 5-003, a bicyclic thymoleptic. *Acta pharmacol et toxicol* 1970 28, 81–96.

Miller L. C. & M. L. Tainter: Estimation of the ED50 and its error by means of logarithmic-probit graph paper *Proc. Soc exp. Biol. Med.* 1944, 57 261 264.

Møller Nielsen, I & K. Neuhold: The comparative pharmacology and toxicology of the trans-isomer of 2 chloro-9-(3 -dimethylaminopropylidene)-thiaxanthene, HCl (chlorprothixene) = N 714 trans and chlorpromazine. *Acta pharmacol. et toxicol.* 1959 15, 335–355.

Petersen, P V N. Lassen, V Hansen, T Huld, J Hjortkjær J Holmblad, I. Møller Nielsen, M. Nymark, V Pedersen, A. Jørgensen & W. Hougs. Pharmacological studies of new series of bicyclic thymoleptics. *Acta pharmacol. et toxicol.* 1966, 24 121 133.

Plym Forshell, G P Schauman, V Hansen, U Dahl Larsen, A. Jørgensen & K. Fredricson Overø Distribution and metabolism of 3,3-dimethyl-1-(3-methylaminopropyl)-1-phenyl-phthalane (Lu 3-010) a bicyclic compound with thymoleptic properties. *Acta pharmacol. et toxicol.* 1968, 26 507–520.

Rubin, B M. H. Malone, M. H. Waugh & J C. Burke: Bioassay of rauwolfia roots and alkaloids. *J Pharmacol. Exp Therap* 1957 120 125–136.

Shipley R. E. & J H. Tilden: A pithed rat preparation suitable for assaying pressor substances. *Proc Soc. exp Biol. Med.* 1947 64 453–455.

Acta pharmacol. et toxicol 1970 **28** 249–257

From the Department of Pharmacology AB Bofors Nobel-Pharma, Mölndal
and the Laboratory of Teratology Karolinska Institutet,
Stockholm, Sweden

Pharmacological Properties of Some Antirheumatic Salicylates

By

G. Åberg and K. S. Larsson

(Received December 22, 1969)

Abstract. The anti-inflammatory effect of acetylsalicylic acid, salicylsalicylic acid, sodium salicylate and salicylamide was investigated by means of the carrageenin-induced oedema-test in rats. Salicylamide in these experiments was found to be significantly less effective than the other compounds tested. With regard to the effect on ^{35}S-sulphate incorporation into calf rib cartilage *in vitro* salicylsalicylic acid was superior to the other compounds as it had an inhibitory effect of 70 % as compared with about 45 % for acetylsalicylic acid and about 35 % for salicylamide. The analgesic activity was investigated using the phenylquinone-method on mice. Acetylsalicylic acid was found to be more active in this respect than the other salicylates. Gastric irritation was studied on the gastric mucosa of guinea-pigs. After sodium salicylate and acetylsalicylic acid administration the mucosa became hyperaemic and in addition acetylsalicylic acid caused numerous erosions in the stomachs. Neither salicylsalicylic acid nor salicylamide gave any significantly increased rate of gastric irritation in the guinea-pigs.

Key-words: Salicylates – anti-inflammatory agents – analgesia.

The anti-inflammatory and analgesic properties are the main factors which determine the value of antirheumatic salicylates. In order to decrease morning stiffness, long biological half lives of the compounds in the body are also desirable. Furthermore it is obvious that the compounds must not give rise to side-effects which might limit the use of the drug in adequate doses. Acetylsalicylic acid has been used as an anti-inflammatory and analgesic drug since the beginning of this century However the biological half-life of acetylsalicylic acid after oral administration to man is short (4 7 hours according to Nordqvist *et al* 1965) and it damages the gastric mucosa (Weiss *et al.* 1961).

In a search for other compounds that would fulfill the requirements for an antirheumatic salicylate better than acetylsalicylic acid, we have studied many salicylate derivatives during the last few years. In this investigation acetylsalicylic acid (ASA), salicylsalicylic acid (SSA), salicylamide (SA) and sodium salicylate (NaSA) have been compared in some laboratory investigations.

Material and Methods

Influence on carrageenin-induced oedema.

Female Sprague-Dawley rats weighing 70–85 g were used in these experiments. The animals were carefully selected and only rats with a normal hind-paw volume of 0.50 ml were used.

The left hind-paw was injected subcutaneously with 0.1 ml of a 1.0 per cent solution of carrageenin 5 min. after the oral administration of 2 ml/100 g body weight of the test compounds in a 1 per cent solution of sodium carboxymethyl cellulose (CMC-Na). Th volumes of the paws were measured 90, 150 270 and 330 min. after the injection of carrageenin. A water-filled graduated cylinder the upper ungraduated part of which had been cut off, was used for measuring the volumes of the paws which were immersed to a mark on the skin over the lateral malleolus (WINTER 1965).

Influence on ^{35}S-sulphate incorporation in vitro

Costal cartilage from recently killed new-born calves was cleaned of perichondrium and sliced in a modified meat-slicing machine (BOSTRÖM & MÅNSSON 1953). The slices measuring 0.4–0.5 mm in thickness, were collected in cold Tyrode's solution containing 0.01 per cent glucose at pH 7.4. A stainless steel cork borer was used to punch out circles 2.4 mm in diameter from the slices.

Six circular pieces of cartilage were incubated in a single flask containing 5 ml of the incubation solution. For each experiment there were 10 control flasks, containing Tyrode solution only and 20 experimental flasks containing the test substances added to the medium.

The compounds tested were acetylsalicylic acid, salicylsalicylic acid and salicylamide in a final concentration of 6 mM at pH 7–7.5. As sodium hydroxide and hydrochloric acid were added to the Tyrode solution for dissolving salicylsalicylic acid, this incubation medium was run as a special control group.

Incubation was carried out in a shaker water-bath at 37. During incubation the flasks were aerated with a mixture of 93.5 per cent oxygen and 6.5 per cent carbon dioxide. The cartilage circles were incubated for one hour before the addition of the labelled sulphur and then for two hours thereafter. Each flask received 50 µcl of carrier-free ^{35}S-labelled sodium sulphate[1]) in 50 µl of distilled water.

At the end of the incubation period the reaction was stopped by the addition of 1 ml of a 2 per cent solution of monoiodoacetic acid. Ten minutes later this mixture drawn off and replaced with a saturated solution of sodium sulphate. Following overnight storage in sodium sulphate, the cartilage was washed twice with water (1 hour each wash) and dehydrated in alcohol, 70 per cent, 2 hours; 95 per cent, 2 hours; 100 per cent, overnight. The cartilage was subsequently dried in an oven and weighed on a Cahn electrobalance. Two pieces of weighed cartilage were placed in a single vial for digestion and counting. Digestion was accomplished with 0.1 ml of 70 per cent perchloric acid followed by 0.2 ml of 30 per cent solution of hydrogen peroxide (MAHIN & LOFBERG 1966). The vials were then capped and placed in an oven at 60 for 1 hour. Following digestion the vials were allowed to cool to room temperature after which 10 ml of scintillation counting fluid (1000 ml toluene, 1000 ml 2-methoxyethanol and 4 g omnifluor[2]) was added.

---

1) The Radiochemical Centre, Amersham, England.
2) New England Nuclear Co., Boston, Mass., U.S.A.

The samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. Three vials were counted for each incubation flask. The solutions used in this procedure resulted in a counting efficiency of approximately 95 per cent, as determined by internal standardization.

Analgesic activity

Phenylquinone tests according to HENDERSHOT & FORSAITH (1959) were performed on female NMRI mice weighing 22 24 g. The test compounds were suspended in a 1 per cent solution of carboxymethyl cellulose and administered orally (0.25 ml/10 g body weight) 30 min. before the intraperitoneal injection of the phenylquinone solution (0.01 ml/g body weight of a 2 per cent solution).

The total number of "writhings" in groups of 6 animals was counted cumulatively for 20 min. and the analgesic activity was expressed as the percental inhibition of "writhings".

Gastric irritation.

This method has been described in detail in an earlier report (ÅBERG 1966) in which part of the present material was presented. Guinea-pigs weighing 300-600 g were fasted for 18 hours before the experiment. They were then randomly dosed orally with the test compounds suspended in 1 per cent CMC Na. As well as the substances mentioned above, a buffered suspension of Ca-acetylsalicylate (bamyl® disprill® nobegan®) was tested. Drug concentrations were adjusted to give each animal dose volume of 0.5 ml/100 g weight. The animals were sacrificed by being stunned and bled three hours after the drug had been administered. The stomachs were excised, opened along the greater curvature and on a "blind" basis the number of "microhaemorrhages" was counted under low magnification with a binocular microscope.

Results

Influence on carrageenin-induced oedema.

As seen from table 1 there was no difference in the anti-inflammatory effect against carrageenin-induced oedema between ASA, SSA and NaSA in the doses tested. The oedema reducing effects for all these substances were clearly already demonstrated within 90 minutes and lasted during the whole test period. The anti-inflammatory effect of SA was significantly less than that of the other compounds.

Influence on ^{35}S-sulphate incorporation in vitro

The results of the *in vitro* ^{35}S-sulphate uptake in calf rib cartilage from six different experiments show clearly that SSA is the most potent inhibitor of the salicylates used (table 2). In all the experiments but number 3 there were statistically significant differences between SSA and ASA ($P < 0.01$). The inhibition for SSA and ASA was 70 per cent and 45 per cent, respectively. The inhibitory effect of SA was about half that of SSA.

Table 1

Anti-inflammatory effects of salicylates against carrageenin-induced oedema.

Treatment		Number of animals tested	Paw volume *before* carrageenin ml ± S. E. M.	Paw volume at different times after administration of the drug			
Substance	Dose p. o. mg/kg			90 min. ml ± S. E. M.	150 min. ml ± S. E. M.	270 min. ml ± S. E. M.	330 min. ml ± S. E. M.
Vehicle	–	10	0.50 ± 0.00	0.94 ± 0.05	1.08 ± 0.05	1.15 ± 0.04	1.14 ± 0.04
ASA	200	10	0.50 ± 0.00	0.66 ± 0.02***	0.68 ± 0.01 **	0.75 ± 0.03	0.83 ± 0.02**
SSA	200	10	0.50 ± 0.00	0.67 ± 0.03**	0.71 ± 0.02	0.77 ± 0.03**	0.79 ± 0.03
SA	200	10	0.50 ± 0.00	0.77 ± 0.03	0.91 ± 0.05	1.05 ± 0.05	1.04 ± 0.07
NaSA	200	10	0.50 ± 0.00	0.68 ± 0.01	0.74 ± 0.02***	0.81 ± 0.01**	0.84 ± 0.02*

Anti-inflammatory effects of the test compounds on rat paws that were made oedematous by local injection of 0.1 ml of 1.0 per cent carrageenin solution. The significance of the observed effects in relation to the control values has been marked in the table with = $P < 0.05$ ** = $P < 0.01$, *** = $P < 0.001$.

Table 2

The inhibitory effects of salicylates on ^{35}S-sulphate incorporation in calf rib cartilage.

Experiment no.	Per cent inhibition of ^{35}S incorporation ± S. E. M. ASA	(n)	SSA	(n)	SA	(n)
1	47.0 ± 4.2	(5)	63.2 ± 0.8	(5)	31.3 ± 1.8	(4)
2	40.0 ± 5.7	(4)	66.2 ± 1.8	(4)	39.8 ± 3.3	(5)
3	69.3 ± 4.2	(4)	71.6 ± 0.7	(5)	37.3 ± 1.5	(5)
4	48.7 ± 2.3	(5)	73.1 ± 0.2	(5)	41.6 ± 3.5	(5)
5	39.8 ± 4.6	(5)	68.8 ± 1.1	(5)	34.4 ± 1.4	(5)
6	20.4 ± 9.3	(5)	76.4 ± 1.4	(5)	27.1 ± 1.5	(5)
mean ± S.E.M.	44.7 ± 3.2	(28)	70.0 ± 0.9	(29)	35.4 ± 1.3	(29)

The inhibitory effects of ASA, SSA and SA at 6 mM concentration expressed as per cent of mean values for the control groups.

Table 3

Inhibitory action of salicylates against phenylquinone induced "writhings"

Treatment Substance	Dose p.o. mg/kg	Number of tests (6 mice in each test)	Inhibition of "writhings" per cent ± S. E. M.
ASA	50	11	40.9 ± 6.1
	100	33	73.5 ± 2.3
SSA	100	12	28.6 ± 3.7
	200	4	56.0 ± 13.0
SA	100	4	26.8 ± 10.2
	200	4	54.5 ± 6.0
NaSA	100	6	28.5 ± 3.2

Analgesic activity

The inhibitory action on phenylquinone-induced "writhings" as seen from table 3 is most effectively exerted by ASA. An oral dose of 100 mg/kg ASA is two to three times more effective than SSA, SA and NaSA. Even a dose of 50 mg/kg of ASA is more efficient than a 100 mg/kg dose of SSA, SA and NaSA respectively

Table 4

Effect of salicylates on the gastric mucosa of guinea-pigs.

Treatment: Substance	Treatment: Dose p. o. mg/kg	Number of animals tested	Number of animals with erosions	Number of erosions in stomachs with lesions Mean ± S. E. M.
Vehicle	—	25	2	Two in each animal
ASA	100	25	25	31.9 ± [illegible].7 (Hyperaemic mucosa)
Ca-ASA	100	25	25	10.4 ± 1.0 (Hyperaemic mucosa)
SSA	100	25	7	2.4 ± 0.9
SA	100	25	13	2.4 ± 0.3
NaSA	100	25	23	5.0 ± 0.5 (Hyperaemic mucosa)

Mean number of erosions in stomachs of guinea-pigs 3 hrs after administration of the drugs.

Gastric irritation.

After oral administration to fasting guinea-pigs ASA induced a hyperaemic gastric mucosa and numerous erosive lesions in the stomachs (table 4). After NaSA the stomachs were also hyperaemic, but the number of erosions was much less than after ASA. Neither SSA nor SA caused any significant macroscopic untoward effects on the gastric mucosa of the guinea-pigs. The soluble Ca-ASA, according to this test, gave somewhat less gastric irritations than ASA, but the compound was nevertheless very irritating.

Discussion

For the screening of anti-inflammatory drugs many experimental methods have been described (WINTER 1965). None of the methods alone is sufficient to detect the actions of a drug on all inflammatory reactions and indeed no single drug is capable of depressing all these reactions. The type of anti-inflammatory drugs designed is consequently dependent on the tests used in laboratory screening.

The carrageenin oedema test was chosen in the present investigation since many antirheumatic drugs efficiently reduce this type of induced oedema (WINTER 1965). There is also a good correlation between the results from

carrageenin oedema tests and the clinical effectiveness in chronic rheumatic disease (SPECTOR & WILLOUGHBY 1968). According to this test ASA, SSA and NaSA were all found to be more effective than SA.

Salicylates are among the different antirheumatic and anti-inflammatory drugs which have been found to inhibit mucopolysaccharide synthesis in cartilage, and heart valves *in vitro* as measured by the incorporation of ^{14}C acetate and glucose and of inorganic ^{35}S-sulphate (see review by WHITEHOUSE 1965)

It is interesting to note that in the present study SSA is the most effective salicylate in inhibiting the ^{35}S-sulphate incorporation in the *in vitro* system. BOSTRÖM & MÅNSSON (1955) in experiments with 4 mM concentration of salicylic acid and its analogues demonstrated that ASA is a more potent inhibitor than SA.

It has been suggested that the inhibitory effect on mucopolysaccharide synthesis by salicylates is due to the interference by the drugs with oxidative phosphorylation (BOSTRÖM *et al.* 1964). Recent studies on the failure of salicylate to reduce the ATP-level in rat paw oedema seem to contradict this hypothesis (KALBHEN & DOMENJOZ 1967) A direct inhibitory effect by salicylate on the enzyme L-glutamine-D-fructose-6-phosphate-aminotransferase, which catalyzes the synthesis of glucosamine-6-phosphate, has been obtained in studies by JACOBSON *et al.* (1964). Further support for the view that glucosamine-6-phosphate formation from fructose-6-phosphate and glutamine is inhibited by salicylate is obtained from the fact that the stimulatory effect of glutamine in this reaction is abolished by sodium salicylate (LARSSON *et al.* 1968 BEAUDOIN *et al.* 1969).

SIEGMUND *et al.* (1957) have demonstrated that intraperitoneal injections of phenylquinone produce "writhings in mice and they also showed that analgesics blocked the "writhings Later HENDERSHOT & FORSAITH (1959) designed the "phenylquinone test which has been universally used for the screening of analgesics. In an attempt to correlate "writhings with "pain CARROLL & LIM (1958) found in rats that the "writhings were abolished by sensory nerve section and by intraperitoneal procaine injections. LIM & GUZMAN (1968) concluded that the receptors for writhings appear to be situated in the gastrointestinal tract and that the afferent impulses pass through the lower brainstem to connections in the posterior lobe of the cerebellum.

Although the phenylquinone test is not a simple pain test – the "writhings are blocked by many drugs which are not analgesics (HENDERSHOT & FORSAITH 1959) – good dose/response relationships are obtained for salicylates and many other analgesics. In the phenylquinone tests reported here ASA was superior to the other salicylates. This is in good agreement with the clinical impression (NORDQVIST 1969 personal communication) that ASA is the best

analgesic salicylate against headache and other acute pain. The pain-mitigating effect of salicylates in rheumatoid arthritis is however not a pure analgesic effect. Because of their antirheumatic activities the compounds decrease the causes of the pain sensation and through this mechanism another and more desirable type of analgesia is achieved.

The gastric effects of ASA and SSA have recently been studied in man (EDMAR 1970). From this investigation it was evident that the gastric effects in man were very similar to those in guinea-pigs. As pointed out before (ÅBERG 1966) *we believe that the subjective feeling of discomfort after the* intake of ASA is correlated to the effects on the gastric mucosa. This does not mean, however, that all patients with gastric erosions and bleeding necessarily have subjective symptoms of gastric irritation.

Acknowledgements

These studies were partly supported by grants from The Swedish Medical Research Council (14 P – 993 – 05). We are indebted to Drs. Fritz Henn and Lennart Hartbon for valuable criticism of the manuscript.

REFERENCES

Beaudoin, A. R., H. Boström, U. Friberg & K. S. Larsson. The effect of sodium salicylate on the glutamine – and glucosamine-induced stimulation of S^{35}-sulphate incorporation *in vitro*. *A kir f Kemi* 1969. 30. 523–527.

Boström, H., K. Berntsen & M. W. Whitehouse: Biochemical properties of anti-inflammatory drugs II. Some effects on sulphate-^{35}S metabolism *in vivo*. *Biochem. Pharmacol.* 1964. 13. 413–420.

Boström, H. & B. Månsson: Factors influencing the exchange of the sulphate group of the chondroitin sulphuric acid of cartilage *in vitro*. *A kir f Kemi* 1953, 6, 23–37.

Boström, H. & B. Månsson. The action of salicylates and related compounds on the sulphate exchange of chondroitin sulphuric acid. *J. Pharm.* (London) 1955, 7 185–190.

Carroll, M. N. J. & R. K. S. Lim. Mechanism of phenylquinone writhings. *Fedn. Proc. Fedn. Am. Soc. exp. Biol.* 1958, 17. 357 p.

Edmar. D. Effects of salicylates on the gastric mucosa as revealed by roentgen examination and the gastrocamera. *Acta Radiol.* 1970, in press.

Hendershot, L. C. & J. Forsaith. Antagonism of the frequency of phenylquinone-induced writhings in the mouse by weak analgesics and nonanalgesics. *J. Pharmacol. Exp. Ther.* 1959. 125. 237–240.

Jacobson, B., H. Boström & K. S. Larsson. The effect of sodium salicylat on hexosamine synthesis in eviscerated mouse fetuses. *Acta Chem. Scand.* 1964, 18, 818–821.

Kalbhen, D. A. & R. Domenjoz. The effect of phenylbutazone and sodium salicylate on the adenosintriphosphate level in rat paw oedema. *Bull. Chim. Therap.* 1967. 5 375–377.

Larsson, K. S., H. Boström & G. Juthedén. Influence of *in vivo* salicylate treatment on 35S-sulphate *in vitro* incorporation in mesenchymal tissue. *Arkiv f. Kemi* 1968, 29 389–394.

Lim, R. K. S. & F. Guzman. Manifestations of pain in analgesic evaluation in animals and man. In. *Pain*. Ed. Soulairac, Cahn & Charpentier. Academic Press Inc., London and New York 1968, 119–152.

Mahin, T. D. & R. T. Lofberg: A simplified method of sample preparation for determination of tritium, carbon-14, or sulphur 35 in blood or tissue by liquid scintillation counting. *Anal. Chem.* 1966, 16, 500–509.

Nordqvist, P., J. G. L. Harthon & R. Karlsson. Omsättningskinetik för salicylsalicylsyra, acetylsalicylsyra och natriumsalicylat hos människa. *Nord. Med.* 1965, 57 1074–1076.

Siegmund, E. A., R. A. Cadmus & G. Lu. A method for evaluating both nonnarcotic and narcotic analgesics. *Proc. Soc. exp. Biol. Med.* 1957, 95, 729–731.

Spector, W. G. & D. A. Willoughby: The testing of anti-inflammatory drugs. In. *The Pharmacology of Inflammation*, Ed. Spector & Willoughby. The English Universities Press, London, 1968, 111–116.

Weiss, A., E. R. Pitman & E. C. Graham. Aspirin and gastric bleeding, gastroscopic observations, with review of literature. *The Amer. J. Med.* 1961, 31 266–278.

Whitehouse, M. W.: Some biochemical and pharmacological properties of anti-inflammatory drugs. *Arzneimittelforsch.* 1965, 8, 321–429.

Winter, C. A.: Anti-inflammatory testing methods: Comparative evaluation of indomethacin and other agents. In: *International symposium on non-steroidal anti-inflammatory drugs*. Ed. Garattini & Dukes. Excerpta Medica foundation, Milan, 1965, 190–202.

Åberg, G. Der Effekt der Acetylsalicylsäure und der Disalicylsäure auf die Ventrikelschleimhaut bei Meerschweinchen. Arzneimittelforsch. 1966, 16, 898–899.

Acta pharmacol. et toxicol 1970 28 258–264

From the Institute of Aviation Medicine, Royal Norwegian Air Force,
Oslo, Norway

ECG-Changes Induced by Phenothiazine Drugs in the Anaesthetized Rat

By

Asbjørn Langslet

(Received December 22, 1969)

Abstract. The ECG has been recorded in barbiturate anaesthetized rats during intravenous infusion of levomepromazine, chlorpromazine, prochlorperazine and perphenazine. All the drugs decreased impulse-generation and propagation in the heart. The magnitude of the changes in heart rate, PQ- QRS- and QT-time was dose dependent, and the relative order of potency of the drugs was as follows: 1. perphenazine and prochlorperazine, 2. chlorpromazine and 3. levomepromazine. Perphenazine, prochlorperazine, chlorpromazine and levomepromazine in 2.5×10^{-6} M concentration increased the PQ-time to about 130 %, 128 %, 122 % and 117 % respectively of the PQ-time before infusion. The magnitude of the changes in rate, QT- and QRS-intervals was in the same range. At higher concentrations, all the drugs caused AV-block of variable degree. Qualitatively these changes are similar to those induced by the drugs in the isolated perfused rat heart, quantitatively however these *in vivo* findings do not correspond to the *in vitro* findings recently described by the present author. Levomepromazine which *in vitro* was unique in changing the ECG-pattern at much lower concentrations than the other phenothiazine derivatives, is less potent than perphenazine, prochlorpromazine and chlorpromazine *in vivo*. Possible explanations for this discrepancy are discussed.

Key words. Phenothiazines – heart.

The ECG-changes produced in experimental animals and in human subjects given tranquillizing phenothiazine (phentiazinum NFN) derivatives are well known. (Reinert & Hermann 1960; Teitelbaum 1963; Wilson & Reese 1964; Hollister & Kosek 1965; Ban & St Jean 1965; Poulsen 1965; Linqvarde 1967) The most common changes are prolongations of the PQ-QRS-QT-intervals, reduced sinus rhythm preceded by tachycardia, partial AV-block, total AV-block, and arrhythmias mainly of ventricular origin, which in some cases have proved fatal.

Recently Landmark *et al.* (1969) and Langslet (1969) showed that the

tranquilizing phenothiazine derivatives produced similar ECG-changes in the isolated perfused rat heart. Following the addition of different phenothiazine derivatives to the perfusion medium they found a dose dependent decrease in rate and impulse conduction velocity and in higher doses, also various degrees of AV-block. It was concluded that these drugs had a direct action on the heart possibly by changing the properties of the cardiac cell membrane.

The cardiac effect of these drugs *in vivo* however might differ from that observed *in vitro* in two respects. Firstly the drugs might affect the heart indirectly by acting on the nervous system, as well as acting directly on the cardiac muscle cells. Secondly differences in distribution and metabolism may change the relative order of potency. In the following report the cardiac effects of a series of phenothiazine derivatives given *in vivo* are described and compared with the *in vitro* studies previously performed. It is shown that even in the same species, the relative order of potency *in vivo* differs from that observed *in vitro*

Material and Methods

Male Wistar Albino rats weighing about 500 g were used in all the experiments. The animals were anaesthetized with pentobarbital (mebumalum NFN) 20 mg/kg body weight intraperitoneally. Ten min. after the injection the animals were asleep and showed normal respiration. Subcutaneous ECG electrodes were then placed as described by FRASER *et al.* (1967). The abdomen was then opened and the inferior caval vein exposed. A stainless steel cannula was placed in this vein. At this time the ECG was controlled and only animals with a normal ECG (FRASER *et al.* 1967) were included in this series.

The drugs, levomepromazine, perphenazine, prochlorperazine, and chlorpromazine dissolved in isotonic NaCl in equimolar concentrations were infused continuously at constant slow rate (0.2 ml/min.) until death of the animal. ECG tracings were continuously made during the experimental period on a Mingograph 14 recorder. Isotonic NaCl solution was used in the control experiments and infused in volumes equivalent to the largest volumes of drug solutions used and at a similar slow rate. Three series f experiments were carried out with each drug and three control experiments were also performed. The molar concentrations were calculated as if the drugs were distributed equally in the total body fluid of the rats.

Results

In the control experiments no change in ECG could be observed, and the respiration was not altered during saline infusion. During the continuous infusion of the drugs, no increase in cardiac rate was observed, but all the drugs caused a dose dependent decrease in rate with sinus rhythm (table 1)

Table 1

The table shows the reductions in heart rate caused by prochlorperazine, perphenazine, chlorpromazine and levomepromazine at different concentrations. The drugs were continuously infused intravenously at constant speed and ECG-tracings continuously made. The rate after infusion of a given dose was calculated in per cent of the rate before the infusions was started. The molar concentrations of the drugs were calculated as if they had been distributed equally in the total body fluid of the animals.

Experiment no.	Drug	Heart rate in per cent of control at the drug concentration indicated below						
		6×10^{-6} M	1×10^{-6} M	1.5×10^{-5} M	2.5×10^{-5} M	4×10^{-5} M	6×10^{-5} M	8×10^{-5} M
1	Prochlorperazine	100	93	79	68	48		
2		100	96	81	65	47		
3		100	96	81	70	50		
4	Perphenazine	100	100	96	84			
5		100	100	97	84			
6		100	100	95	87			
7	Chlorpromazine	100	100	100	90	65	35	24
8		100	100	100	90	62	32	23
9		100	100	100	89	63	33	25
10	Levomepromazine	100	100	100	100	86	71	53
11		100	100	100	100	84	74	54
12		100	100	100	100	87	72	54

Table 2

The table shows the prolongations of the PQ-interval caused by prochlorperazine, perphenazine, chlorpromazine and levomepromazine at different concentrations. The drugs were continuously infused intravenously at constant speed and ECG-tracings continuously made. The PQ-interval after infusion of a given dose was calculated in per cent of the PQ-interval before the infusion was started. The molar concentrations of the drugs were calculated as if they had been distributed equally in the total body fluid of the animals.

Experiment no.	Drug	PQ-interval in per cent of control at the drug concentration indicated below					
		6×10^{-9}	1×10^{-8}	2.5×10^{-8}	4×10^{-8} M	6×10^{-8}	8×10^{-8} M
1	Perphenazine	100	113	130			
2		100	114	129			
3		100	117	133			
4	Prochlorperazine	100	110	130	141		
5		100	109	127	138		
6		100	109	127	142		
7	Chlorpromazine	100	106	122	132	156	167
8		100	108	125	133	157	169
9		100	105	121	133	157	167
10	Levomepromazine	100	101	118	125	148	159
11		100	104	121	127	148	161
12		100	103	120	128	145	158

Their relative order of potency in this respect calculated on a molar basis, was. 1 prochlorperazine 2. perphenazine 3 chlorpromazine 4 levomepromazine All the drugs caused a dose dependent prolongation of the PQ-QRS-QT interval (table 2) Their relative order of potency in this respect, calculated on a molar basis, was 1 perphenazine, 2. prochlorperazine, 3 chlorpromazine 4 levomepromazine In higher doses all the drugs caused partial AV block which turned into total block on increasing the doses. The relative order of potency of the drugs in causing AV-block, calculated on a molar basis, was 1 perphenazine, 2. prochlorperazine, 3 chlorpromazine, 4 levomepromazine. In some of the experiments ectopic atrial pacemakers without tachycardia or fibrillation were observed. In none of the experiments were supraventricular or ventricular tachycardia or fibrillation induced, and none of the experimental animals died from ventricular arrest. The lethal doses of all the drugs tested caused severe respiratory distress terminating in respiratory failure Their relative order of potency in this respect, calculated on a molar basis, was. 1 perphenazine, 2. prochlorperazine 3. chlorpromazine, 4 levomepromazine (table 3).

Table 3

The table shows the doses of levomepromazine chlorpromazine, perphenazine, and prochlorperazine that cause respiratory arrest in 12 experimental animals. The drugs were continuously infused intravenously at constant speed until death of the animal. 3 experiments were carried out with each of the drugs. The molar concentrations of the drugs were calculated as if they had been distributed equally in the total body fluid of the animals.

Experiment no	Drug	Dose that causes respiratory arrest
1		1.4×10^{-4} M
2	Levomepromazine	1.6×10^{-4} M
3		1.7×10^{-4} M
4		8.5×10^{-5} M
5	Chlorpromazine	9.5×10^{-5} M
6		1×10^{-4} M
7		3.2×10^{-5} M
8	Perphenazine	2.9×10^{-5} M
9		3.4×10^{-5} M
10		5.5×10^{-5} M
11	Prochlorperazine	4.9×10^{-5} M
12		5.8×10^{-5} M

Discussion

In a recent paper LANGSLET (1969) has described a dose dependent decrease in cardiac impulse generation and conduction velocity in the isolated perfused rat heart induced by different phenothiazine derivatives.

During these *in vitro* conditions, the relative order of potency of the drugs in reducing cardiac rate and conduction velocity calculated on a molar basis was 1 levomepromazine, 2. perphenazine, 3 prochlorperazine, 4 chlorpromazine. In the present investigation similar dose dependent changes in ECG-pattern were found in the living anaesthetized rat given phenothiazine derivatives. However the relative order of potency of the drugs in changing the ECG-pattern, calculated on a molar basis, was different during *in vivo* conditions, namely 1 perphenazine, 2. prochlorperazine 3. chlorpromazine, 4 levomepromazine. Levomepromazine which *in vitro* was unique in changing the ECG at much lower concentrations than the other phenothiazine derivatives, was less potent than perphenazine, prochlorperazine and chlorpromazine in this respect *in vivo* As far as perphenazine, prochlorperazine and chlorpromazine are concerned, their relative order of potency is similar *in vitro* and *in vivo*

It is reasonable to imagine that the dose dependent changes in ECG produced by the phenothiazine derivatives investigated *in vivo* which are essentially similar to those produced by the drugs *in vitro* must be due to a direct action of the drugs on the heart. Furthermore it is possible that *in vivo* these direct cardiac effects of the drugs are modified, and that the magnitude of this modification depends on the chemical structure of the phenothiazine derivatives. The nature of the modifying factors, cannot however be elucidated by our experiments. But, differences between the drugs plasma binding, – tissue binding – and distribution, as well as different effects of the drugs on the hormonal and nervous control of the heart, must be involved and warrant further investigation.

In spite of the fact that phenothiazine derivatives can produce cardiac complications, all the drugs tested caused death of the animals by arresting respiration. This finding is in accordance with the observations of COURVOISIER *et al* (1953) in the case of chlorpromazine. In our experiments the animals were anaesthetized with pentobarbital which might have altered the cardiac action of the phenothiazine derivatives. Furthermore the phenothiazine derivatives may potentiate the effect of pentobarbital on respiration. Species differences must also be taken into account.

Acknowledgements

I would like to thank Mr K. Wehn for his technical assistance, and the head of the institute, Dr F V Lorentzen for his help.

REFERENCES

Ban, T A. & A. St. Jean. Electrocardiographic changes induced by phenothiazine drugs. *Amer Heart J* 1965 70 575–576.

Courvoisier S., J Fournel R. Ducrot, M. Kolsky & P Koetschet. Pharmacodynamic properties of 3-chloro-10(3'-dimetylaminopropyl) Phenothiazine HCl (RD4560). Experimental study of a new compound used in potentiating anesthesia and in artificial hiberbation. *Arch. Intern. Pharmacody* 1953 92, 305–361

Fraser R. S. *et al.* Electrocardiogram in the normal rat. *Journal of pplied Physiology* 1967 23 401–402.

Hollister L. E. & J C. Kosek. Sudden death during treatment with phenothiazine derivatives. *J Amer med. Ass.* 1965 192, 1035–1038.

Landmark, K., A. Glomstein & I Øye: The effect of thioridazine and promazine on the isolated working rat heart. *Acta pharmacol. et to icol* 1969 27 173–182.

Langslet, A.. Changes in coronary flow and ECG in the isolated perfused rat heart induced by phenothiazine drugs. *Acta pharmacol. et toxicol.* 1969 27 183–192.

Lingjerde, O EKG-forandringer kardiale rytmeforstyrrelser og mors subita under fenothiazinebehandling. *T norske Laegeforen.* (The journal of the Norwegian Medical Association) 1967 87 90–94.

Poulsen, H.. Grenblokk som komplikasjon til klorpromazinbehandling. *Ugeskr Laeg.* 1965 127 720–721

Reinert, R. E. & C. G Hermann Unexplained deaths during chlorpromazine therapy *J nerv ment Dis.* 1960, 131 435–442.

Teitelbaum, I.: Electrocardiographic changes associated with phenothiazine. *Lancet* 1963, *I* 115

Wilson, I. & J C. Reese: Simultaneous death in schizophrenic twins. *Arch. gen. Psychiat.* 1964 11 377 384

Acta pharmacol. et toxicol. 1970, 28, 265–269

From the Central Laboratory and the Departments of Epileptic Diseases,
The Filadelfia Colony Dianalund, Denmark (Chiefs: J Danielsen,
V Faghang-Frederiksen and C. Munk Plum)

The Influence of Folic Acid on Phenytoin (DPH) Metabolism and the 24-Hours Fluctuation in Urinary Output of 5-(p-hydroxyphenyl)-5 phenyl-hydantoin (HPPH)

By

O Vendelin Olesen and O. Nygaard Jensen

(Received September 3, 1969)

Abstract Five patients with phenytoin (DPH) induced subnormal serum *L* casei folat activity were treated for a period of 5 months with 20 mg of folic acid daily in order to investigate the effect of this treatment on the quantitative 24-hours output of 5-(p-hydroxyphenyl)- 5 phenyl-hydantoin (HPPH) and on the serum phenytoin levels. During the period of folic acid treatment an average decrease of 12 % in the mean HPPH output was found, and four out of the five patients showed corresponding fall in the serum phenytoin levels. In each patient a large variation in the 24-hours output of HPPH was found, and at least seven consecutive 24-hours urine samples during each of two given periods are necessary in order to demonstrate any change in the HPPH output. It is suggested that treatment with massive doses of folic acid alters the metabolism of phenytoin towards an unknown metabolic route.

Key words Diphenylhydantoin – phenytoinum – folic acid – human experimentation – urinary clearance.

In recent years several independent investigations have shown that patients treated with phenytoin (DPH) have a subnormal serum folate concentration (KLIPSTEIN 1964 IBBOTSON *et al.* 1967 REYNOLDS 1968 JENSEN & OLESEN 1969) In all cases the folic acid concentration was measured by means of a microbiological method using *l* (lactobacillus) casei as a test organism. Since, however many folic acid derivatives are growth factors and have varying effects, the terms folate or folic acid concentration should be replaced by *l* casei activity

An unselected group of in-patients treated with phenytoin showed a significantly lower *l* casei activity in the serum and whole blood than a control group from the same hospital given the same diet (JENSEN & OLESEN 1970). However controlled studies have shown that the phenytoin induced subnormal *l* casei activity is of no clinical significance as the patients exhibit no other signs of folate deficiency (HORWITZ *et al* 1968 JENSEN &

OLESEN 1969) On the other hand, this phenomenon is of considerable theoretical interest. Patients with phenytoin-induced subnormal *l* casei activity show a significantly lower excretion of formiminoglutamic acid after histidine load as compared with a matched control group (JENSEN & OLESEN 1969). This observation suggests an effect on the metabolic processes which require folic acid as a coenzyme

If folic acid is necessary for the action of the oxidizing liver enzymes, the metabolism of phenytoin may cause an increased demand for the vitamin and consequently possible a decreased, serum *l* casei activity

The aim of the present study has been to investigate the effect of massive daily doses of folic acid on the serum levels of phenytoin and on the excretion of its main metabolite 5-(p-hydroxyphenyl)-5 phenyl hydantoin (HPPH), (BUTLER 1957 MAYNERT 1960) in patients with a phenytoin-induced subnormal *l* casei activity

Material and Methods

Five patients were selected, all hospitalized and treated with phenytoin for at least 10 years. They were well known to the staff for their readiness to cooperate.

Patient A. 31 year old male, weight 63 kg. 4-hours phenytoin dose 150 + 200 mg.

Table 1

Daily 5-(p-hydroxyphenyl)-5-phenyl-hydantoin excretion. In the first period the results from both 24-hours and night urines (calculated by means of extrapolation) are shown. The differences in the outputs before and after treatment for 5 months with 20 mg of folic acid per day are indicated.

Percentage of 24-hours phenytoin dose excreted as HPPH

Patient	Before treatment with folic acid: 4-hours urine		Before treatment with folic acid: Calculated from the night urine			At the start of treatment			After 5 month of treatment			Difference
	Mean	S.E.M	Mean	S.E.M.	d. f	Mean	S.E.M.	d. f	Mean	S.E.M.	d. f.	
A	80.5	± 1 67	80 1	± 2.72	14	80.4	± 4.26	15	72.3	± 1.00	15	8.2
B	86.9		73.1	± 4.18	14	70.9	± 4.59	13	63.7	± 4 68	13	23.2
C	87.2	± 1.62	84 4	± 3.03	14	88.3	± 4.89	15	74.1	± 3.88	15	13.1
D	79.5	± 2.18	81 0	± 3.07	14	82.6	± 2.03	15	75.9	± 2.81	15	3.6
E	73.2	± 2.41	80.5	± 2.94	14	61.9	± 6.19	14	62.5	± 7.97	13	10.7
Average	81.5	± 2.31	79.8	± 3 19		76.8	± 4.39		69 7	± 4 07		11.8

S. E. M. = Standard error of the mean.
d. f = Degrees of freedom.

serum *L casei* activity 1.2 μg/l. B. 24 year old female, 62 kg, phenytoin dose 150 + 200 mg, serum *L casei* activity 0.9 μg/l. C: 28 year old male, 78 kg, phenytoin dose 150 + 150 mg, serum *L casei* activity 2.0 μg/l. D 36 year old male, 76 kg, phenytoin dose 100 + 200 mg, serum *L casei* activity 1.1 μg/l, and patient E. 38 year old female, 52 kg, phenytoin dose 100 + 100 mg serum *L casei* activity 1.7 μg/l.

The patients were given 20 mg of folic acid per day orally for 5 months.

24-hours urines were collected daily for three periods each of 14–16 days, namely before, at the start of folic acid treatment and after treatment for 5 months.

Collection of urine from patients may involve difficulties. The most reliable is the urine voided in the morning and thus excreted during the night. In order to check the reliability of the urine sampling, the urine excreted during the day and the urine excreted during the night were collected and analyzed for HPPH separately. If the hourly output during the night corresponded to the hourly output during the day it was accepted that no urine had been lost. No such loss could be detected (table 1).

All blood samples for determination of serum DPH levels were drawn from fasting patients at bout 7 a. m. every second week.

The urinary content of HPPH and serum DPH levels were determined (in duplicate) by the methods previously described (OLESEN 1967a, 1967b & 1968).

The serum *L casei* folate activity was determined by Professor Hoff Jørgensen, Biochemical Department, Royal Dental College, Copenhagen. The normal range was 2 10 μg/l serum calculated as folic acid.

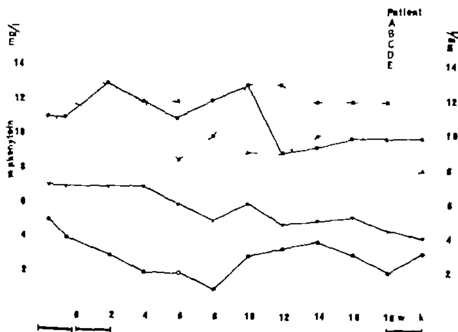


Fig. 1. Serum phenytoin (DPH) levels during folic acid administration 20 mg per day started at zero. The horizontal lines indicate the periods during which urine was collected.

Results

From table 1 it is seen that folic acid administration induced a *slight* decrease in the urinary output of HPPH. An average decrease of 12 per cent was found in the mean HPPH excretion.

In spite of the large daily variations the differences between the means before and after treatment for 5 months with folic acid were found to be statistically significant for patient A, B and C. In the last two patients only a tendency was found in this direction.

Fig. 1 shows a trend to a decrease in the serum phenytoin levels during the experimental period in four of the five patients.

In fig. 2 the *l. casei* activity of the packed red cells of patient A, B and C during the period of folic acid treatment is shown. From the figure it is seen that maximum activity was achieved in the course of 80–90 days.

Discussion

During the period of folic acid treatment a decrease in the 24-hours excretion of HPPH was found. Four out of the five patients also showed a

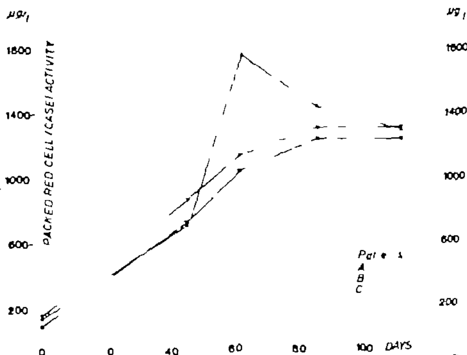


Fig. 2. Packed erythrocyte *L. casei* activity calculated as folic acid in the patients A, B and C. Folic acid administration was started at zero line.

decrease in the serum phenytoin level. Monthly determinations of 180–305 samples of serum phenytoin from patients in this hospital during the year in which this investigation was carried out showed no seasonal variation. It is thus unlikely that the changes observed were due to such a variation.

In view of the large variation in the 24-hours output of the metabolite no conclusion can be reached about the quantitative changes in the output of HPPH when this is based on single 24-hours urinary outputs. The variation is not due to errors in the determination of HPPH or to incomplete urine collection.

The results indicate that treatment with massive doses of folic acid influences the metabolism of phenytoin. As the urinary excretion of HPPH is found to decrease and grossly follows the serum phenytoin level the results suggest that folic acid administration to some extent at least alters the phenytoin metabolism from hydroxylation to an unknown metabolic pathway which normally accounts for 20–40 % of the ingested dose.

REFERENCES

Butler T. C. The metabolic conversion of 5,5-diphenyl-hydantoin to 5-(p-hydroxyphenyl)-5-phenylhydantoin. *J Pharmacol. Exp Therap* 1957 119 1 11.

Horwitz, S. J F A. Klipstein & R. E. Lovelace: Relation of abnormal folat metabolism to neuropathy developing during anticonvulsant drug therapy *Lancet*, 1968, *I* 1 563–565

Ibbotson, R. N., Dilena, B A. & J M. Horwood. Studies on deficiency and absorption of folates in patients on anticonvulsant drugs. *Aust. A n. Med* 1967 16 144–150.

Jensen, O. N. & O. V Olesen. Folic acid and anticolvulsive drugs. *Arch. Neurol.* 1969 21 208–214.

Jensen, O N & O V Olesen. Folate concentrations in psychiatric patients. *Acta psychiat. scand.* 1970 45, 289–294.

Klipstein, F A.: Subnormal folates and macrocytosis associated with anticonvulsant drug therapy *Blood* 1964, 23, 68–86.

Maynert, E. W The metabolic fate of diphenylhydantoin in the dog, rat and man. *J Pharmacol Exp Therap.* 1960, 130 275–284

Olesen, O V A simplified method for extracting phenytoin from serum, and more sensitive staining reaction for quantitive determination by thin-layer chromatography *Acta pharmacol. et toxicol.* 1967a, 25 123–126.

Olesen, O V Determination of phenobarbital and phenytoin in serum by ultraviolet spectrophotometry *Scand. J Clin Lab Invest* 1967b 20 63–69

Olesen, O. V Determination of 5(p-hydroyphenyl)-5-phenyl-hydantoin (HPPH) in urine by thin-layer chromatography *Acta pharmacol et toxicol.* 1968, 26, 222 228.

Reynolds, E. H. Mental effect of anticonvulsants, and folic acid metabolism. *Brain* 1968, 91 197–214

Acta pharmacol et toxicol 1970 28 270–282.

From the Department of Pharmacology
University of Copenhagen, Denmark

The Effects of Guanethidine, Pre- and Postganglionic Nerve Division on the Rat Superior Cervical Ganglion Cholinesterases and Protein

By

Jens Jensen-Holm and Per Juul

(Received January 15, 1970)

Abstract. Administration of guanethidine (20 mg/kg for 8–14 days) to rats induced a pronounced reduction in the activity of specific and non-specific cholinesterase accompanied by an increase of protein content of the superior cervical ganglion. These effects were not changed by pre- or postganglionic nerve division. Consequently a direct ganglionic effect of guanethidine is assumed. Isoprenaline and propranolol had no effects on the parameters measured, and these drugs did not influence the action of guanethidine. Hence the ganglionic changes induced by guanethidine do not seem to be due to a beta-stimulating activity. A few other drugs were investigated: furosemide, pentolinium, atropine, phentolamine, desipramine, and alpha-methyldopa, but none of these caused any significant changes in the ganglionic cholinesterases and protein. Bethanidine eventually induced cholinesterase changes similar to those of guanethidine, but further studies are needed.

Key-words. Guanethidine – ganglia, autonomic – cholinesterase – proteins.

Prolonged administration of guanethidine to rats causes a marked reduction in the activities of specific (AChE) and non-specific (ChE) cholinesterases in sympathetic ganglia (JENSEN-HOLM 1967). An increase in the size and protein content of the ganglia was also found. In a preliminary report (JENSEN-HOLM & JUUL 1968) these changes were studied by means of cholinesterase histochemistry and histology and it was found that the loss of AChE and ChE was due to disappearance of the enzymatic activity from the ganglionic nerve fibres as well as from the cytoplasm of the nerve cells. The increase in the size of the ganglia was due to infiltration of small cells. These findings are described in detail in a following paper (JENSEN HOLM & JUUL 1970).

It has been the main purpose of the present study to investigate and localize the effects of guanethidine as described above by simultaneous unilateral preganglionic nerve division or postganglionic axotomy. Furthermore, since

guanethidine has been reported to possess a certain beta-adrenergic stimulating activity (BHARGAVA *et al.* 1966 BRUNNER *et al.* 1965), the combined effects of guanethidine and a beta-stimulating or a beta-blocking agent have been studied. Finally the possible ganglionic effects of some other drugs have also been investigated.

Material and Methods

Male Wistar rats weighing 180–240 g at the beginning of the experiment (average 204 g) were used. Unilateral preganglionic nerve division or postganglionic axotomy of the superior cervical ganglion was carried out under ether anaesthesia. The effectiveness of the operations was controlled by the presence of narrowing of the palpebral fissure and of the pupil. Guanethidine monosulphate (10 mg/ml in saline) was administered intraperitoneally in fixed volumes: 25 100, 250 and 500 µl per rat. The doses in the tables are presented in mg/kg (mean of weights at the initiation and at the end of the experiment). The injections were started 2–7 days after the operations. The animals were exsanguinated under ether anaesthesia and the superior cervical ganglia excised after dissection and kept at − 20 until the analyses were carried out.

Cholinesterases.

The ganglia were homogenized in a glass homogenizer at ice-cold conditions. To one ganglion 400–600 µl of saline was added. For the determination of the activity of AChE and ChE 100 µl of the homogenate was used (often duplicate analyses) and the same amount was used for protein determination.

The activities of AChE and ChE were determined by a titrimetric procedure (JENSEN-HOLM 1965) at pH 7.4 and 38 and the figures refer to the activity obtained with 1 mM of acetylcholine iodide. The final reaction volume was 1.5 ml consisting of normal saline, 100 µl of the homogenate and the solution of substrate. Acetylcholine was added stepwise to final concentrations of 1 and 10 mM, and the activities obtained with these two substrate concentrations corrected for spontaneous substrate hydrolysis and blank value were used for the calculation procedure (for details see JENSEN-HOLM 1965). In superior cervical ganglia from control rats about 75 % of the corrected activity was due to AChE and 25 % due to ChE when expressed at 1 mM of acetylcholine. If the values were given at 10 mM of acetylcholine, the total activity here would be of the same magnitude, but AChE and ChE would each now constitute about 50 %

Protein determination.

The protein content was determined according to the method of LOWRY *et al.* (1951) slightly modified. 100 µl of the homogenate was treated with 50 µl 0.2 N-NaOH The final reaction volume, 5650 µl, contained all of the homogenate. The sample was read at 750 nm. The results were calculated from standard curve simultaneously obtained.

Drugs used.

Guanethidine monosulphate (ismelin®) 10 mg/ml dissolved in saline.
Isoprenaline (isoproterenol) sulphate 50 mg/ml
Propranolol (propranololum NFN) chloride (inderal®) 1 mg/ml
Pentolinium hydrogen tartrate (ansolysen®) 5 mg/ml
Furosemide (lasix®) 10 mg/ml
Atropine sulphate 10 mg/ml

Phentolamine methanesulphonate 10 mg/ml
Alpha-methyldopa 10 mg/ml
Desipramine (desmethylimipramine chloride, pertofran®) 12.5 mg/ml
Bethanidine sulphate (regulin®) 20 mg/ml.

The injections were carried out once daily and in a few instances twice daily. Most of the solutions were injected intraperitoneally (i. p.), and in a few cases subcutaneously (s. c.) (see text to the tables). The statistical calculations are based on Student's t-test.

Results

I. Guanethidine pre and postganglionic nerve division.

In table 1 the results of administration of guanethidine at doses of approximately 20 mg/kg daily for one or two weeks on the AChE, ChE, and protein contents are presented. On the side indicated it can be seen whether preganglionic nerve division or postganglionic axotomy has been carried out. *Control rats were given saline. There were no differences between the right* and left ganglia with regard to AChE, ChE, and protein content in untreated, unoperated rats.

14 days after *postganglionic axotomy* only AChE was reduced by 30 % as compared to the unoperated side, and the ChE was reduced by 50 %. When *guanethidine* was administered during the last 8 days of the experiment, AChE on the unoperated side was reduced by 40 %. On the operated side AChE was reduced by 60 % ($P < 0.05$) as compared to results from the operated untreated side. Similar results were obtained for ChE. In a single case a rat was killed 4 weeks after a postganglionic axotomy had been performed. Here AChE was 20 % and ChE 73 % of the values of the unoperated side.

12 days after *preganglionic nerve division* AChE and ChE were reduced by approximately 30 % ($P < 0.05$). After 24 days the AChE and ChE were reduced by approximately 40 % ($P < 0.005$). When *guanethidine* was administered simultaneously as indicated, the activities were further reduced. After treatment for 14 days AChE per ganglion on the operated side was as small as 12 % ($P < 0.001$) as compared to values from the unoperated control side, while the ChE was 25 % ($P < 0.001$).

II. Variation in the doses of guanethidine

In table 2 the AChE and ChE, and the protein content after guanethidine administration for 14 days at different dose levels are presented. The upper part (A) is concerned with rats, in which in addition a preganglionic nerve division was performed on the left side. The results indicate that 1 and 4 mg/kg daily of guanethidine caused no changes in the content of AChE and ChE. After administration of the highest dose (22 mg/kg) a marked reduction in AChE and ChE ($P < 0.01$) was found followed by an increase in the

Table I

Specific (AChE) and non-specific (ChE) cholinesterase activities in the rat superior cervical ganglia expressed in nanomoles per minute. The content of protein per ganglion is given in μg. Previous preganglionic nerve division (pre-R or pre-L) or postganglionic axotomy (post-L) was performed unilaterally on the side indicated (R = right, L = left). S. D. values are presented in brackets. Guanethidine monosulphat or saline was administered intraperitoneally daily for one or two weeks as indicated. n = number of rats.

Drug	Daily dose per kg	Administration period	Days from operation to sacrifice	n	Side	Unoperated side per ganglion nmol/min. AChE	Unoperated side per ganglion nmol/min. ChE	protein μg	Operation	Operated side per ganglion nmol/min. AChE	Operated side per ganglion nmol/min. ChE	protein μg
saline	2 ml	6.–13. day	14	3	R	57.4 (11.8)	13.0 (4.4)	133 (33)	post-L	39.6 (12.2)	6.2 (2.0)	157 (67)
guanethidine	20 mg	6.–13. day	14	3	R	34.6 (14.8)	9.1 (1.8)	218	post L	16.0 (7.0)	3.2 (4.1)	190 (60)
saline	2 ml	5. 11 day	12	3	R	46.4 (4.6)	20.5 (3.4)	152 (21)	pre-L	33.8 (5.3)	13.6 (3.9)	208 (82)
guanethidine	4 mg	5. 11 day	12	4	R	26.4 (4.8)	7.5 (2.7)	186 (64)	pre-L	13.8 (5.9)	8.2 (2.0)	220 (31)
saline	2 ml	8. 21 day	24	4	L	69.1 (7.1)	25.3 (6.4)	242 (58)	pre R	39.9 (6.7)	14.2 (3.4)	184 (91)
guanethidine	20 mg	8. 1 day	24	5	L	22.7 (7.1)	7.7 (2.7)	235 (19)	pre R	8.3 (6.0)	6.4 (3.1)	356 (162)

Table 2

AChE, ChE, and protein in the rat superior cervical ganglia after daily administration intraperitoneally of guanethidine monosulphate for two weeks at different doses as indicated. With regard to the upper half of the table (A) preganglionic nerve division was carried out on the left side 4 days before the start of the injections. With regard to part B no operation was performed. The ganglia from the left side were used for histology and cholinesterase histochemistry (see JENSEN-HOLM & JUUL 1970).

	Drug	Daily dose per kg	Administration period	Days from operation to sacrifice	n		Unoperated side, per ganglion, nmol/min., AChE	Unoperated side, per ganglion, nmol/min., ChE	Unoperated side, protein µg		Operated side, per ganglion, nmol/min., AChE	Operated side, per ganglion, nmol/min., ChE	Operated side, protein µg
A	saline	2 ml	5. 18. day	19	4	R	53.8 (9.4)	18.8 (4.3)	153 (54)	pre-L	3[illegible].7 (1[illegible].4)	11.5 (3.6)	142 (35)
	guanethidine	1 mg	5 18. day	19	4	R	45.7 (8.6)	15.6 (4.9)	132 (31)	pre-L	37.8 (8.0)	12.0 (5.8)	189 (53)
	guanethidine	4 mg	5 18. day	19	4	R	61.5 (15.2)	17.4 (3.5)	183 (51)	pre-L	36.8 (5.7)	14.4 (4.7)	205 (39)
	guanethidine	22 mg	5 18. day	19	4	R	19.0 (8.1)	8.8 (1.4)	230 (80)	pre-L	11.0 (2.2)	4.5 (0.5)	237 (79)
B	saline	2 ml	14 days		4	R	80.1 (16.0)	24.7 (9.6)	204 (100)	no operation			
	guanethidine	1 mg	14 days		3	R	70.3 (2.5)	29.9 (2.6)	173 (44)	no operation			
	guanethidine	4 mg	14 days		4	R	63.8 (17.3)	15.8 (5.2)	153 (42)	no operation			
	guanethidine	11 mg	14 days		4	R	52.9 (21.9)	17.7 (3.5)	165 (57)	no operation			
	guanethidine	21 mg	14 days		4	R	38.4 (16.8)	12.1 (3.0)	174 (5)	no operation			

Table 3

AChE, ChE, and protein in the rat superior cervical ganglia. Guanethidine monosulphate was administered intraperitoneally for 10 days at the average dose indicated. 26 days after discontinuation of the drug the gangli were excised and investigated. The ganglia from the two sides were analysed together (R + L). AChE and ChE values are expressed per ganglion and per mg of protein.

Drug	Daily dose per kg	Duration of administration days	Duration discontinuation days	n		per ganglion nmol/min. AChE	per ganglion nmol/min. ChE	protein µg	per mg of protein nmol/min. AChE	per mg of protein nmol/min. ChE
saline	2 ml	10	26	4	R + L	57.9 (10.0)	21.0 (3.7)	163 (22)	354 (45)	129 (12)
guanethidine	20 mg	10	26	8	R + L	45.1 (12.4)	16.4 (4.0)	225 (42)	199 (35)	73 (15)

protein content. In the lower part (B) the results from another experiment are shown. Four different dose levels were used for the same period of time. The animals were not operated upon. The results obtained seem to indicate a dose-dependent response although a significant depression of both AChE and ChE (about 50 %) was found only in animals treated with 21 mg/kg of guanethidine ($P < 0.05$). In this experiment no increase in the protein content following guanethidine was found. The ganglia from the left side were used for histology and cholinesterase histochemistry and the results are published elsewhere (JENSEN HOLM & JUUL 1970).

By the method used for protein determination the amount of guanethidine present in the ganglion following administration of 20 mg/kg for 14 days was found too small to give a measurable additional colour reaction. This amount of guanethidine did not influence the activity of cholinesterases as determined under the conditions described.

III *Recovery phase*

Guanethidine 20 mg/kg was administered for 10 days and the controls were given the same volume of saline (table 3). After discontinuation of the drug for 26 days the rats were killed. The AChE and ChE activities are expressed both per ganglion and per mg of protein. The activities of the two enzymes per ganglion were then found to be only 22 % lower than those of the corresponding controls. However, when calculated per mg of protein they were 44 % lower ($P < 0.001$). The content of protein was above that of the control level ($P < 0.02$).

IV *Guanethidine, isoprenaline and propranolol*

In table 4 the results are shown of experiments with guanethidine in combination with isoprenaline and propranolol. A preganglionic nerve division was carried out on the left side. In the upper part (A) of the table the results of drug administration for 7 days are presented. Guanethidine reduced the activity of AChE and ChE on both sides ($P < 0.01$ except for ChE on the operated side, which was not significantly reduced). These reductions were not affected by the doses used of simultaneously administered isoprenaline or propranolol. The administration of the two latter drugs separately at different dose levels (A and B) and for a longer period, or used together in the same doses as in the combination with guanethidine (A) produced no effects.

V *Other drugs*

In a few experiments other drugs were administered in order to demonstrate whether they could influence the sympathetic ganglia with regard to cholinesterase activity and protein content. The results are presented in table 5. None of the drugs caused significant changes either in enzyme activity or in protein content.

AChE, ChE, and protein in the rat superior cervical ganglia after isoprenaline (subcutaneously), propranolol (intraperitoneally) guanethidine (intraperitoneally) or a combined administration as indicated, in part A for 7 days, in part B for 14 days. Preganglionic nerve division was carried out on the left side (pre-L) 3 days before the start of drug administration.

	Drug	Daily dose per kg	Administration period	Days from operation to sacrifice	n		Unoperated side: per ganglion, nmol/min. AChE	Unoperated side: per ganglion, nmol/min. ChE	Unoperated side: protein µg		Operated side: per ganglion, nmol/min. AChE	Operated side: per ganglion, nmol/min. ChE	Operated side: protein µg
A	saline	2 ml	4.–10. day	11	3	R	46.4 (4.6)	20.5 (3.4)	152 (21)	pre-L	33 8 (5.5)	13 6 (3.9)	208 (82)
	isoprenaline	23 mg	4. 10. day	11	3	R	46.1 (13.3)	20.5 (2.8)	201 (31)	pre L	45.3 (17.0)	19 4 (3.6)	228 (57)
	propranolol	4 mg	4. 10. day	11	4	R	59 2 (17 4)	22.7 (6.2)	172 (43)	pre-L	43.6 (10 0)	18.7 (4.2)	207 (54)
	guanethidine	4 mg	4. 10. day	11	4	R	26.4 (4.8)	7.5 (2.7)	186 (64)	pre-L	13.8 (5.9)	8.2 (2.0)	220 (31)
	isoprenaline guanethidine	26 mg 25 mg	4. 10. day	11	3	R	34.5 (6.9)	12.9 (1.6)	239 (35)	pre-L	7.5 (2.8)	9.9 (3.8)	212 (54)
	propranolol guanethidine	5 mg 23 mg	4. 10. day	11	3	R	34.5 (12.8)	11.6 (7 4)	253 (131)	pre-L	11.9 (3.6)	6 6 (2.8)	253 (41)
	isoprenaline propranolol	23 mg 5 mg	4. 10 day	11	4	R	55 4 (7.0)	16.9 (6.4)	150 (19)	pre-L	25 1 (7.8)	12.3 (4 4)	165 (32)
B	saline	2 ml × 2	3. 17 day	18	5	R	54.2 (14.0)	11.0 (2.3)	104 (24)	pre-L	33.8 (5.9)	9.8 (3.0)	116 (27)
	isoprenaline	19 mg × 2	3. 17 day	18	5	R	52.0 (13.6)	14.2 (5 9)	117 (31)	pre-L	37.4 (13.5)	11 6 (6.1)	121 (54)
	propranolol	5 mg × 2	3. 17 day	18	5	R	45 6 (22.2)	10 0 (4.5)	91 (27)	pre-L	29.6 (5 9)	7.9 (4.6)	95 (25)

Table 5

AChE, ChE, and protein in the rat superior cervical ganglia after administration of the different drugs listed. The values are expressed as the percentage of the corresponding controls. No statistically significant changes were found.

Drug	Daily dose mg/kg	Route of administration	Duration of administration days	n	Percentage of control value per ganglion		
					AChE	ChE	protein
atropine	20	i. p.	11	3	89.7	110.9	91.5
atropine ...	60	i. p.	11	3	95.0	121.2	109.0
α-methyldopa	20	s. c.	10	3	115.2	70.4	102.5
α-methyldopa ...	50	s. c.	10	3	109.1	87.2	100.0
pentolinium ...	11	i. p.	10	4	101.3	111.4	101.1
furosemide	23	i. p.	11	4	103.3	104.8	106.5
phentolamine	15	i. p.	10	6	93.8	87.0	87.3
desipramine ...	5	s. c.	10	3	95.6	87.0	76.3

Discussion

One of the main purposes of the first part of the present investigation has been to show whether the marked reduction in the activity of AChE and ChE in the superior cervical ganglia found in rats treated with guanethidine could be a consequence of its blocking action on the postganglionic sympathetic neurones, a direct ganglionic action, or an unknown action exerted centrally to the ganglia.

Postganglionic axotomy alone causes a complete loss of AChE and ChE from the ganglion cells within two days according to HÄRKÖNEN (1964) and also from the preganglionic fibres as based on histochemical methods. This has also been shown by BROWN (1958). Using a similar method the enzymatic loss has been confirmed by JENSEN HOLM & JUUL (1970). By means of the quantitative titrimetric determination of cholinesterase activity used in the present investigation, however, a different result has been obtained. From table 1 it is obvious that AChE and ChE 2 weeks after postganglionic axotomy still show quite high activities (for AChE about 70 % and for ChE 50 % of the activity still persists), while histochemical investigations on ganglia from a parallel group of animals showed hardly any cholinesterase activity independent of variations in the histochemical procedure. Therefore it must be emphasized that the operations were effective, and this is also supported by the eye symptoms observed in the rats. This discrepancy between the biochemical and histochemical findings will be further discussed in

a following paper (Jensen-Holm & Juul 1970). The biochemical results agree well with those obtained by McLennan (1954) who found that AChE was reduced by between 23 and 76 % (on an average about 50 %) 3 weeks following axotomy In disagreement with our results McLennan stated that this reduction only concerned AChE since "pseudo-ChE" did not appear to be present in this tissue. ChE however has been shown to be present in the rat superior cervical ganglion, as described both in the present and in the following paper – as well in the nerve fibres as in the cytoplasm of the ganglion cells. Other investigators have also shown the presence of ChE in the rat superior cervical ganglion (Dhar 1958 Klingman *et al.* 1968) Dhar (1958) found a decrease in AChE 3 weeks after postganglionic axotomy of approximately 50 % and a decrease in ChE of only 15 % indicating that regenerative processes may have occurred.

Preganglionic nerve division leads to a similar loss of activity of the two enzymes as found after postganglionic axotomy as determined by the titrimetric method used. The average decrease in activity of AChE and ChE following preganglionic denervation of 30-40 % compares well with the results of Dhar (1958), who found a reduction of the enzymatic activity of 43 % and 25 % respectively 2 weeks after denervation. Brown (1958) and Härkönen (1964) found that AChE disappeared completely from the nerve fibres within two days, but not from the ganglion cells. This is confirmed in a following paper (Jensen-Holm & Juul 1970)

Guanethidine administration. As previously shown (Jensen-Holm 1967 Jensen-Holm & Juul 1968) guanethidine in a dose of approximately 20 mg/kg daily for one or two weeks causes a pronounced loss of both AChE and ChE. From table 1 it appears that postganglionic axotomy and preganglionic nerve division do not prevent the effects of guanethidine, since the drug after both types of operations still reduces the enzymatic activity This seems to indicate that the drug has a direct ganglionic effect, although it is not possible to exclude the possibility that additional central or peripheral actions are involved in the results obtained, when guanethidine only is used.

One problem to be solved is whether the decrease in enzymatic activity is due to a decreased rate of production or an increased rate of disappearance or both. Malmquist & Oates (1968) have shown that guanethidine inhibits the oxidative phosphorylation in rat liver mitochondria *in vitro* Härkönen (1964) has shown that following postganglionic axotomy an accumulation of AChE is observed in the distal part of the proximal stump two days after the operation. Another problem is the importance of the cholinesterases in the function of the sympathetic ganglia.

In table 2 the AChE and ChE activities in the superior cervical ganglia after different doses of guanethidine are shown. Doses below 10 mg/kg are not effective, whereas higher doses reduced the cholinesterase

the majority of the experiments the protein content of the ganglia is elevated following guanethidine corresponding to the increased size of the ganglia. The difference between part A and B in table 2 in this respect cannot be explained.

Guanethidine administered at the highest dose levels indicated, caused a slight decrease in weight gain of the rats. After lower doses a normal growth rate was found (ZAIMIS 1966).

Table 3 indicates the results altered at discontinuation of treatment. It appears from the figures that the activities of AChE and ChE after discontinuation of treatment for almost 4 weeks are still below the control values, especially when expressed per mg of protein, the content of which is significantly above the control value. Therefore it must be assumed that the recovery following the doses and period of administration used is a very slow process and/or that partly irreversible changes may have occurred.

BRUNNER *et al.* (1965) have shown that pronethalol antagonizes the antihypertensive effect of guanethidine in Goldblatt hypertensive rats. On the basis of these experiments the second part of the present investigation was planned in order to study the possible interference of isoprenaline and propranolol with the effect of guanethidine on the superior cervical ganglion. The results are presented in table 4. Isoprenaline and propranolol given separately or together did not seem to influence the activity of AChE and ChE, and the effect of guanethidine was not affected by the simultaneous administration of these drugs. On the basis of the above mentioned findings it may be concluded that the ganglionic changes due to guanethidine cannot be a consequence of a beta-stimulating effect. With regard to propranolol this could be due to insufficient doses. The doses of isoprenaline used are highly effective in the rat. POHTO (1966) found that even lower doses of this drug were effective in inducing a pronounced enlargement of the serous salivary glands. In the present experiment an enlargement to several times the control weight of the salivary glands was found. Propranolol slightly reduced the total weight of the salivary glands, but did not decrease the effect of isoprenaline. The AChE and ChE findings will be published separately.

The third part of the present investigation deals with the possible effect of other drugs on the ganglionic cholinesterases. The results are presented in table 5. JENSEN HOLM (1967) has shown that daily injections of saline (2 ml/kg for 6–14 days) caused a slight reduction in the content of protein and a small increase in AChE and ChE. Investigations were thus performed to determine whether a diuretic agent (furosemide) might have the opposite effect, but this was not found to be the case. The drug was certainly given in an effective dose, as the rats lost about 20 g in weight within the first few hours of each injection. Pentolinium as a blocking agent of ganglionic transmission and atropine which blocks the atropine-sensitive cholinoceptive

sites of the autonomic ganglia did not affect AChE and ChE. Phentolamine which blocks the peripheral alpha-receptors, and desipramine which blocks the amine re-uptake did not influence the values in the superior cervical ganglion nor did alpha-methyldopa. A few experiments with bethanidine in the very large doses of 60–110 mg/kg daily for 10 days seem to indicate an action similar to that of guanethidine, i. e. a reduction in the cholinesterase activities, but presumably without affecting the protein content. These experiments will therefore be repeated together with an investigation of other drugs possessing a postganglionic neurone blocking activity particularly compounds with a chemical composition close to that of guanethidine.

Acknowledgements

Guanethidine monosulphate (Ismelin®) was generously supplied by Danish CIBA. The analyses were undertaken by Miss Tove Gøricke.

REFERENCES

Bhargava, K. P. B. P. Jain & K. K. Tangri: Mechanism of the central hypotensive action of guanethidine. *Brit. J. Pharmacol.* 1966, 27, 491–496.

Brown, L. M.: Cholinesterase in the superior cervical ganglion of the rat after preganglionic denervation and axotomy. *J. Physiol.* (London) 1958, 142, 7P–8P.

Brunner, H. P. R. Hedwall & M. Meier: Influence of an adrenergic beta-receptor blocking agent on the effect of various hypotensive agents in the hypertensive rat. *Experientia* 1965, 21, 231–232.

Dhar, S. K.: Cholinesterase in decentralized and axotomized sympathetic ganglia. *J. Physiol.* (London) 1958, 144, 27P.

Härkönen, M.: Carboxylic esterases, oxidative enzymes and catecholamines in the superior cervical ganglion of the rat and the effect of pre- and postganglionic nerve division. *Acta physiol. scand.* 1964, 63, suppl. 237.

Jensen-Holm, J.: A titrimetric method for separate determination of specific and non-specific cholinesterase activity in the absence and presence of irreversible inhibitors (Paraoxon and DFP). *Acta pharmacol. et toxicol.* 1965, 23, 73–84.

Jensen-Holm, J.: Cholinesterase and protein changes in sympathetic ganglia after guanethidine. *Acta pharmacol. et toxicol.* 1967, 25, suppl. 4, 74.

Jensen-Holm, J. & P. Juul: Changes in the rat superior cervical ganglion induced by guanethidine (histology and cholinesterase histochemistry). *Brit. J. Pharmacol.* 1968, 34, 211P.

Jensen-Holm, J. & P. Juul: The effects of guanethidine, pre- and postganglionic nerve division on the rat superior cervical ganglion: cholinesterases and catecholamines (histochemistry) and histology. *Acta pharmacol. et toxicol.* 1970, 28, 283–298.

Klingman, G. L., J. D. Klingman & A. Poliszczuk: Acetyl- and pseudocholinesterase activities in sympathetic ganglia of rats. *J. Neurochem.* 1968, 15, 1121–1130.

Lowry, O. H., N. J. Rosebrough, A. L. Farr & R. J. Randall: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 1951, 193, 265–275.

the majority of the experiments the protein content of the ganglia is elevated following guanethidine corresponding to the increased size of the ganglia. The difference between part A and B in table 2 in this respect cannot be explained.

Guanethidine administered at the highest dose levels indicated, caused a slight decrease in weight gain of the rats. After lower doses a normal growth rate was found (ZAIMIS 1966)

Table 3 indicates the results altered at discontinuation of treatment. It appears from the figures that the activities of AChE and ChE after discontinuation of treatment for almost 4 weeks are still below the control values, especially when expressed per mg of protein the content of which is significantly above the control value. Therefore it must be assumed that the recovery following the doses and period of administration used is a very slow process and/or that partly irreversible changes may have occurred.

BRUNNER *et al.* (1965) have shown that pronethalol antagonizes the antihypertensive effect of guanethidine in Goldblatt-hypertensive rats. On the basis of these experiments the second part of the present investigation was planned in order to study the possible interference of isoprenaline and propranolol with the effect of guanethidine on the superior cervical ganglion. The results are presented in table 4 Isoprenaline and propranolol given separately or together did not seem to influence the activity of AChE and ChE, and the effect of guanethidine was not affected by the simultaneous administration of these drugs. On the basis of the above mentioned findings it may be concluded that the ganglionic changes due to guanethidine cannot be a consequence of a beta-stimulating effect. With regard to propranolol this could be due to insufficient doses. The doses of isoprenaline used are highly effective in the rat. POHTO (1966) found that even lower doses of this drug were effective in inducing a pronounced enlargement of the serous salivary glands. In the present experiment an enlargement to several times the control weight of the salivary glands was found. Propranolol slightly reduced the total weight of the salivary glands, but did not decrease the effect of isoprenaline. The AChE and ChE findings will be published separately

The third part of the present investigation deals with the possible effect of other drugs on the ganglionic cholinesterases. The results are presented in table 5 JENSEN-HOLM (1967) has shown that daily injections of saline (2 ml/kg for 6–14 days) caused a slight reduction in the content of protein and a small increase in AChE and ChE. Investigations were thus performed to determine whether a diuretic agent (furosemide) might have the opposite effect, but this was not found to be the case. The drug was certainly given in an effective dose, as the rats lost about 20 g in weight within the first few hours of each injection. Pentolinium as a blocking agent of ganglionic transmission and atropine which blocks the atropine-sensitive cholinoceptive

Acta pharmacol. et toxicol. 1970, 28, 283–298.

From the Department of Pharmacology, University of Copenhagen, Denmark

The Effects of Guanethidine, Pre- and Postganglionic Nerve Division on the Rat Superior Cervical Ganglion: Cholinesterases and Catecholamines (Histochemistry), and Histology

By

Jens Jensen-Holm and Per Juul

(Received January 15, 1970)

Abstract: Guanethidine administered intraperitoneally in a dose of 20 mg/kg for 5 to 14 days induced a considerable reduction of histochemically demonstrabl specific and non-specific cholinesterases. The loss initially involved the cytoplasm of the ganglion cells, but following prolonged administration there was a complete loss which also involved the nerve fibres. After guanethidine 1–25 mg/kg an increasing degree of chromatolysis of the sympathetic ganglion cells was observed accompanied by infiltration of small cells. The increased number of small cells caused an increase in ganglionic size of approximately 50 % (guanethidine 20 mg/kg for 14 days). The increased number of small cells mainly consisted of small lymphocyte-like cells, some of which showed pyroninophilia, and of macrophages. Mitoses were not observed, and the number of Schwann cells appeared unchanged. The possibility of an immunological process is discussed. The changes appeared to be specific, as only sympathetic ganglion cells were involved. These changes were partly reversible, though a number of ganglion cells had undergone complete degeneration. The ganglionic noradrenaline was partially depleted by guanethidine. The alterations induced by guanethidine resembled those resulting from postganglionic axotomy, but they developed independently of previously performed pre- or postganglionic nerve division. It is suggested that guanethidine has a direct effect on the ganglion cells.

Key-words: Guanethidine – ganglia, autonomic – histocytochemistry – histology

Previous investigations have demonstrated the acute transient ganglionic blocking activity of guanethidine, whilst no impairment of ganglionic function could be demonstrated following prolonged administration of high doses (Bein 1960 among others). Following prolonged administration of guanethidine Jensen-Holm (1967) and Jensen-Holm & Juul (1968 & 1970) however demonstrated an increase in the size and a decrease in the specific (AChE) and non-specific (ChE) cholinesterase activity of the rat superior cervical ganglion. The catecholamine depleting action of guanethidine has been shown to include the superior cervical ganglion (Sanan & Vogt 1962)

The purpose of the present investigation has been to localize the enzymatic loss at the cellular level and to examine the structural alterations of the rat superior cervical ganglion following guanethidine administration. The changes are compared to those induced by preganglionic nerve division and postganglionic axotomy and the combined effects of guanethidine administration and of previously performed pre- or postganglionic nerve division. A preliminary report of these findings has been published previously (JENSEN-HOLM & JUUL 1968). A pilot histochemical study of the catecholamines is included in the present paper.

Material and Methods

A total of 140 male Wistar rats, aged 7–13 weeks, weighing 170–340 g at the start of the experiment (usually 200–250 g, average 232 g) were used. 34 rats were untreated or received daily intraperitoneal injections of saline 0.5 ml for varying lengths of time. 43 rats were given daily intraperitoneal injections of guanethidine monosulphate 1–25 mg/kg dissolved in saline (usually 10 mg/ml) for 28 days followed by discontinuation of treatment for 1–56 days. In 44 rats unilateral preganglionic nerve division was performed under ether anaesthesia and in 19 rats unilateral postganglionic section was performed. Guanethidine or saline was subsequently administered as described above. The effectiveness of the operations was checked by the presence of narrowing of the palpebral fissure and constriction of the pupil. The animals were killed by exsanguination under ether anaesthesia and the superior cervical ganglia quickly excised after dissection.

Ganglia from 68 rats were examined histologically (214 specimens) and ganglia from 77 rats were examined histochemically (477 specimens for cholinesterases). Ganglia from 6 rats were examined for catecholamine fluorescence (12 specimens). In each experiment specimens from control animals were also included. In the case of pre- or postganglionic nerve division a comparison was also made with the contralateral ganglion.

a. *Weight*

Ganglia freeze-dried for 24 hours were weighed on an electro-balance. Statistical calculations are based on Student's t-test.

b. *Cell counts.*

Cell counting and measuring was performed on photographic enlargements, and in each specimen the cells within an area of 0.05 mm² were counted. The ratio of small cells/ganglion cells was chosen as an indication of the degree of ganglionic changes in order to avoid errors concerning absolute figures, due to changes in ganglionic size, small differences in magnification, and shrinkage due to fixation.

c. *Histology*

The ganglia were fixed in 3.5 % neutral phosphate buffered formaldehyde at 4° for at least 24 hours, embedded in paraffin and then sectioned longitudinally at 4 microns. Routine histological techniques were used, i.e. staining for Nissl substance by means of the gallocyanine-chromalum method (EINARSON 1932) and staining for nerve

fibres by means of the silver proteinate technique of BODIAN (1936). With regard to the other histological techniques used, see text to figures.

The sections were photographed in Leitz Orthoplan microscope. The Köhler illumination principle was used throughout except for the cholinesterase histochemistry where the aperture diaphragm was further reduced t increase the contrast.

d. *Cholinesterase histochemistry*

The ganglia were either sectioned fresh frozen (43 rats) or fixed (34 rats) in 3.5 % neutral phosphate buffered formaldehyde at 4 for 4 hours, followed by storage in 10 % sucrose at 4 overnight. Cryostat sections were cut longitudinally t 10 microns and attached t glass slides followed by air-drying for 15–60 minutes. For the demonstration of cholinesterases a thiocholine-lead-ferrocyanide method modified from the technique described by ERÄNKÖ *et al.* (1967) and based on the original procedure of KARNOVSKY & ROOTS (1964) was used. Preincubation was carried out for 15 minutes at room temperature in moisture saturated chamber in 35 mM tris-(hydroxymethyl) aminomethane buffer at pH 6.1 containing 0.5 mM potassium ferricyanide and 5 mM lead acetate. Incubation was carried out for 20 minutes t room temperature in an identical solution after addition of substrate. Both solutions were filtered through Munktell filter paper no. OOH. As substrates acetylthiocholine iodide and butyrylthiocholine iodide (Fluka) at 3.2×10^{-3} M were used. From each ganglion three specimens were prepared. 1) for staining of both AChE and ChE (acetylthiocholine as substrate without inhibitor), 2) for staining of AChE (acetylthiocholine as substrate, addition t the preincubation solution of Mipafox 10^{-4} M (HOLMSTEDT 1957)), and 3) for staining of ChE (butyrylthiocholine as substrate without inhibitor). Following incubation, the specimens were

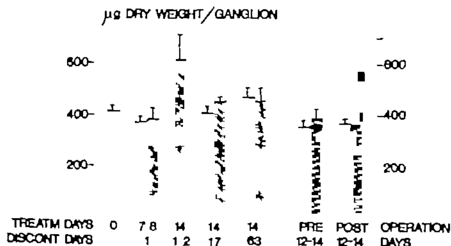


Fig. 1. Weight of freeze-dried superior cervical ganglia from rats treated with guanethidine monosulphate at 20–25 mg/kg for various periods and following cessation of treatment for various periods (left part of the figure) and after unilateral pre- or postganglionic nerve division (right part of the figure). Unhatched columns represent control ganglia, in the right part of the figure the contralateral ganglia. Mean values and S. E. M. are indicated. The figures above the columns indicate the number of ganglia weighed.

washed 3 times in distilled water for 5 minutes, immersed in 1.6 % ammonium sulphide, rinsed in distilled water air-dried for at least 2 hours, and after xylol, mounted in D P X. In some cases counterstaining was performed with 0.1 % toluidine blue. Sections treated with acetylthiocholine in the presence of both Mipafox at 10^{-4} M and ambenonium at 10^{-4} M showed no cholinesterase activity and this was also the case when butyrylthiocholine was used together with Mipafox at 10^{-4} M

e. *Catecholamine histochemistry*

For the demonstration of catecholamines the formaldehyde fluorescence technique described by FALCK & OWMAN (1965) was used.

Results

I *Control ganglia.*

a) *Weight* The weight of the freeze-dried superior cervical ganglia of untreated rats was on an average 413 μg (fig. 1) The solids constituted approximately 20 / of the wet weight.

b) *Cell counts* Calculated per mm² the number of ganglion cells was on an average 640 and the number of small cells 3240 The ratio of small cells/ganglion cells is shown in fig. 2.

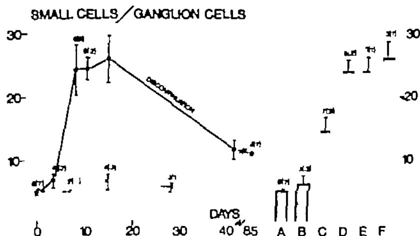


Fig. 2. The ratio small cells/ganglion cells in the superior cervical ganglia from rats treated with guanethidine 20–25 mg/kg (unbroken line) and 10 mg/kg (dotted line) in the left part of the figure. The right part of the figure shows the same ratio in normal ganglia (A), preganglionically denervated ganglia (B), postganglionically axotomized ganglia (C), ganglia from rats treated with guanethidine 20–25 mg/kg for 10 days (D), combination of guanethidine administration and preganglionic denervation (E), and combination of guanethidine and postganglionic axotomy (F). Mean values and S. E. M. are indicated. The figures above the individual points and columns indicate the number of specimens counted, the figures in brackets the number of rats in each group.

c) *Histology* The diameter of the multipolar ganglion cells varied between 20 and 45 microns. Cells with two nuclei were very rarely observed. The number of nucleoli varied between one and three. The granular Nissl substance was evenly distributed throughout the cytoplasm (fig. 3A) By the Bodian staining two types of ganglion cells could be distinguished. one type with a heavily stained and another with a lightly stained nucleus (fig. 4A) The ganglion cells were surrounded by capsular cells, Schwann cells, endoneural connective tissue and endothelial cells, and a number of unidentified small cells. A varying number of nerve fibres ranging from less than 0.5 to more than two microns in diameter was observed, the majority running longitudinally in the ganglion (fig. 4A).

d) *Cholinesterase histochemistry* AChE and ChE were localized in both the ganglion cells and the nerve fibres. In the ganglion cells the staining was confined to the cytoplasm. The staining of the nerve fibres was probably mainly due to staining of preganglionic nerves (fig. 6A-C) The ganglion cells could be divided into three groups according to the intensity of the cholinesterase activity observed. Approximately one third of the cells showed an intense histochemical staining, approximately half of the cells an intermediate, and one tenth a weak reaction. Only a few cells showed no visible cholinesterase activity In most of the ganglion cells the cytoplasm stained more intensely for AChE than for ChE, whereas the nerve fibres showed a relatively stronger staining for ChE than for AChE. Apart from this relative difference, the staining for AChE and ChE was of the same overall intensity under the conditions chosen. The interstitial cells showed only a very faint or no staining. No essential differences were observed between fixed and unfixed specimens, except that the enzymatic localization was sharper in the former

e) *Catecholamine histochemistry* The green fluorescence due to nor adrenaline was present in the cytoplasm of the ganglion cells and in the nerve fibres (fig. 7A). Only a few small cells showed fluorescence.

II. *Preganglionic denervation (decentralization).*

a) *Weight.* There was no significant difference between the average weight of denervated ganglia and the control ganglia (fig. 1)

b) *Cell counts* The number of ganglion cells and small cells was unaffected by denervation (fig. 4B). In fig. 2B the cell ratio is shown.

c) *Histology* There were no visible changes of the Nissl substance (fig. 3B). A number of thick nerve fibres had disappeared, whereas the thin nerve fibres seemed unaffected (fig. 4B).

d) *Cholinesterase histochemistry* A few days after preganglionic denervation characteristic changes were observed. The staining for both choli

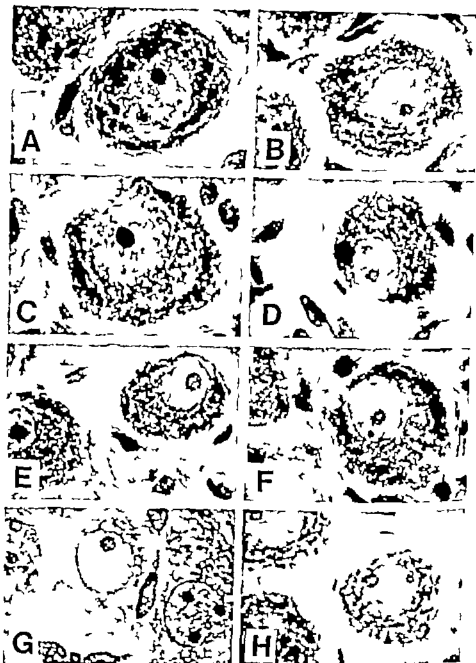


Fig. 3. Rat superior cervical ganglion. Gallocyanine staining for Nissl substance. Magnification × 1000.

A. Control. B. 14 days following preganglionic denervation. C. 10 days following post ganglionic axotomy. D. Guanethidine 1 mg/kg for 14 days. E. Guanethidine 4 mg/kg for 14 days. F. Guanethidine 11 mg/kg for 14 days. G. Guanethidine 20 mg/kg for 10 days. H. Guanethidine 22 mg/kg for 14 days followed by cessation of treatment for 28 days.

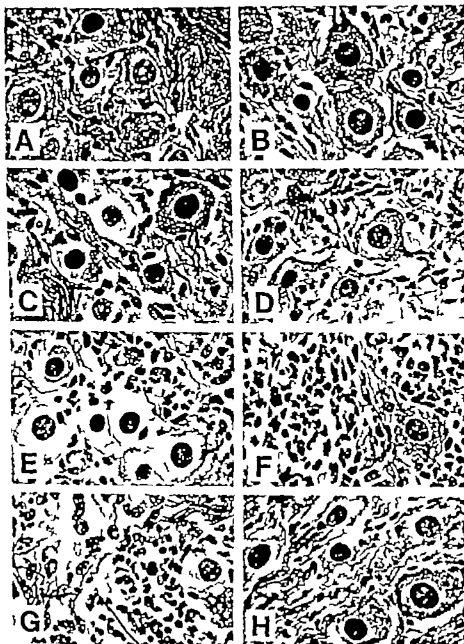


Fig. 4. Rat superior cervical ganglion. Silver proteinat staining after Bodian. Magnification × 400

A. Control. B. 8 days following preganglionic denervation. C. 8 days following post ganglionic axotomy. D. Guanethidine 24 mg/kg for 4 days. E. Guanethidine 25 mg/kg for 8 days. F. Guanethidine 25 mg/kg for 14 days. G. Guanethidine 23 mg/kg for 28 days. H. Guanethidine 22 mg/kg for 14 days followed by cessation of treatment for 28 days.

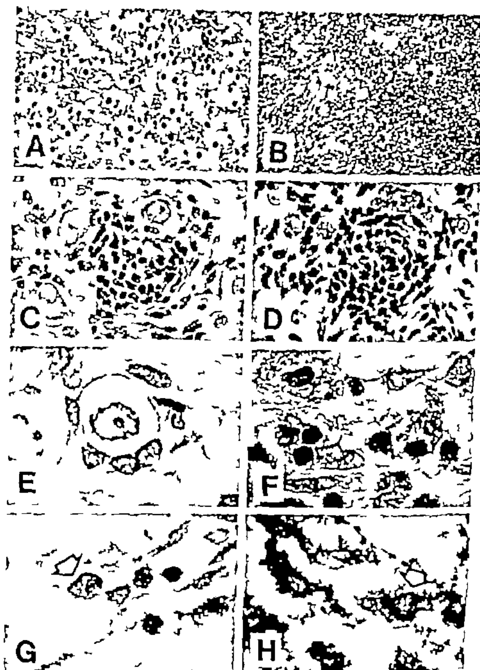


Fig. 5. Rat superior cervical ganglion. Demonstration of small cells.
A. Control (Bodian, × 100). B. Guanethidine 25 mg/kg for 14 days (Bodian; × 100). C. 10 days following postganglionic axotomy (van Gieson-Hansen; × 400). D. Guanethidine 22 mg/kg for 7 days (van Gieson-Hansen, × 400). E and F Guanethidine 20 mg/kg for 10 days (haematoxylin-eosin, × 1000) G Guanethidine 20 mg/kg for 10 days (methyl-green-pyronine × 1000). Arrow indicates pyroninophilic cell. H Guanethidine 20 mg/kg for 10 days (MARSHALL 1956; × 1000). Arrow indicates metallophilic cell.

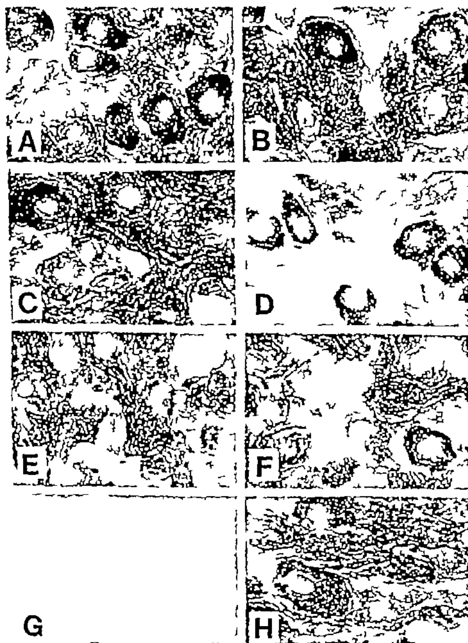


Fig. 6. Rat superior cervical ganglion. Histochemical demonstration of cholinesterases. Magnification × 400

A. Control, AChE. B. Control, ChE. C–H. AChE + ChE. C Control. D. 8 days following preganglionic denervation. E. 4 days following postganglionic axotomy. F Guanethidine 25 mg/kg for 8 days. G Guanethidine 23 mg/kg for 14 days. H. Guanethidine 23 mg/kg for 14 days followed by cessation of treatment for 28 days.

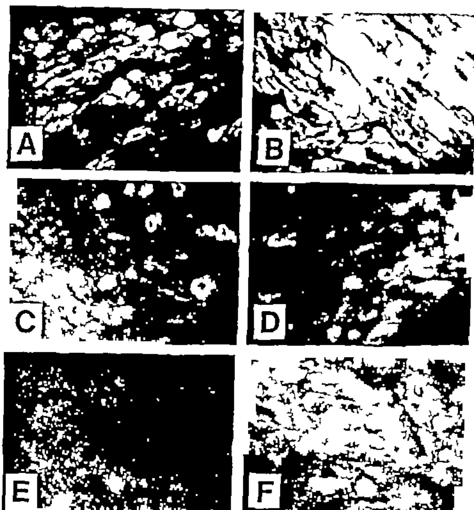


Fig. 7 Rat superior cervical ganglion. Catecholamine fluorescence. Magnification × 250. A. Control. B. 10 *days following preganglionic nerve division.* C. 10 *days following* postganglionic axotomy. D. Guanethidine 20 mg/kg for 10 days. E. Combination of guanethidine and preganglionic denervation. F. Combination of guanethidine and postganglionic axotomy.

ases was now almost exclusively confined to the cytoplasm of the ganglion cells, whereas the activity of the nerve fibres had virtually disappeared (fig. 6D). The staining intensity of the cytoplasm seemed slightly increased in some of the cells and had probably disappeared from others.

e) *Catecholamine histochemistry.* The green fluorescence was increased in the majority of the ganglion cells (fig. 7B).

III. *Postganglionic axotomy*

a) *Weight* The weight of the axotomized ganglia was approximately 50 / greater than that of the control ganglia (fig. 1). This difference was statistically significant ($P < 0.001$).

b) *Cell counts* The number of ganglion cells counted as described above was reduced by approximately one third as compared to the control ganglia ($P < 0.02$) When correction was made for the increased volume of the ganglion however the total number of ganglion cells per ganglion was unchanged. The number of small cells was increased by approximately 50 / ($P < 0.01$). When corrected for the increased volume of the ganglion, the increase was more than 100 / of the total number per ganglion The cell ratio is shown in fig. 2C.

c) *Histology* 5–14 days following postganglionic axotomy a considerable number of ganglion cells showed characteristic retrograde degenerative changes a peripheral dislocation of the nucleus and a partial loss of the Nissl substance, often with only a perinuclear chromatolysis (fig. 3C) Differential counting of the small cells showed that only the number of small round cells (4–7 microns) with dark nuclei and of larger round cells (7–12 microns) with lightly stained nuclei was increased. Both types of cells were 4–5 times more numerous in axotomized gangila than in control ganglia, whereas the number of capsular cells and Schwann cells appeared unchanged. Part of the heavily stained nerve fibres had probably disappeared, but this loss was difficult to evaluate, as only the main postganglionic trunk was divided in order to avoid the ischaemic necrosis which almost invariably results from complete stripping of all postganglionic nerves. This fact must also be taken into consideration when the weight increase and the cell counts are evaluated.

d) *Cholinesterase histochemistry* A considerable loss of activity of both cholinesterases was observed. Initially the loss was mainly confined to the cytoplasm of the ganglion cells (fig. 6E), but subsequently the nerve fibres were also involved. In certain cases – probably following more complete cutting of the postganglionic nerves – no activity could be observed.

e) *Catecholamine histochemistry* A considerable reduction in the fluorescence intensity was observed 10 days following axotomy A few cells with apparently normal catecholamine content were seen (fig. 7C)

IV *Guanethidine administration.*

a) *Weight* Following administration of guanethidine 20–25 mg/kg for 10–14 days the weight of the ganglion had increased to approximately 160 / (fig. 1) of the normal value ($P < 0.05$)

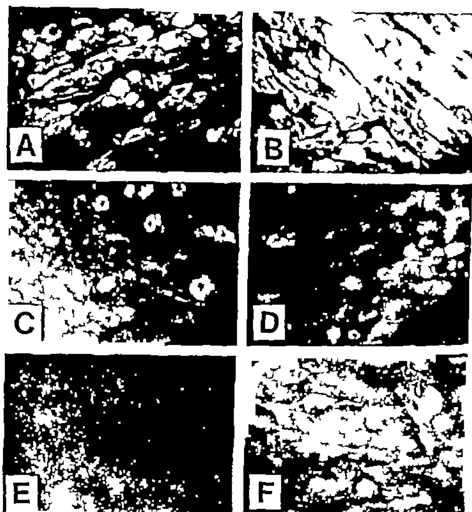


Fig. 7 Rat superior cervical ganglion. Catecholamine fluorescence. Magnification × 250. A. Control. B. 10 days following preganglionic nerve division. C. 10 days following postganglionic axotomy. D. Guanethidine 20 mg/kg for 10 days. E. Combination of guanethidine and preganglionic denervation. F. Combination of guanethidine and postganglionic axotomy.

ases was now almost exclusively confined to the cytoplasm of the ganglion cells, whereas the activity of the nerve fibres had virtually disappeared (fig. 6D). The staining intensity of the cytoplasm seemed slightly increased in some of the cells and had probably disappeared from others.

e) *Catecholamine histochemistry.* The green fluorescence was increased in the majority of the ganglion cells (fig. 7B).

changes of cell counts, histology cholinesterase and catecholamine histochemistry were similar to those induced by guanethidine administration alone (fig. 2 and 7E-F)

VI. *Regeneration phase.*

Following discontinuation of guanethidine administration for up to 56 days a partial regeneration occurred. The cell counts, histology and cholinesterase histochemistry were partly normalized (fig. 2, 3H, 4H, 6H). Complete regeneration was not observed. None of the ganglia in this group were weighed, and catecholamine histochemistry was not performed.

Discussion

The increased size of ganglia following postganglionic axotomy is in agreement with the findings of NAGATA & TSUKADA (1968) This increase is mainly due to the increased number of small cells. The nature of these small cells is obscure. Some of the larger cells (7–12 microns) probably represent macrophages in the process of neuronophagia. A number of these cells show a metallophilic reaction (fig. 5H). The small lymphocyte like cells (5–7 microns) are often located subcapsularly and in small clusters, probably perivascularly (fig. 5C D). The morphology of these cells is similar to that in axotomized ganglia and in ganglia of rats treated with guanethidine. The infiltrating small cells show no fluorescence in the formaldehyde treated specimens and are therefore not identical with the small fluorescent cells described by ERÄNKÖ & HÄRKÖNEN (1965b) NAGATA & TSUKADA (1968) found frequent mitotic figures and considered most of the proliferated small cells observed following postganglionic axotomy to be Schwann cells. They found, however that it was difficult to determine the definite morphology of these cells (ectodermal or mesodermal origin). In the present investigation mitotic figures were not encountered and the number of Schwann cells was not found to be increased either in axotomized ganglia or in ganglia of rats treated with guanethidine. The small lymphocyte-like cells are quite different from the spindle shaped cells described by NAGATA & TSUKADA (1968) Some of these cells showed pronounced pyroninophilia (fig. 5G). LAMPERT (1965) has described an accumulation of small cells in the central nervous system of rats with experimental allergic encephalomyelitis. He observed perivascular accumulations of mononuclear cells spreading diffusely into the adjacent parenchyma. These cells were distinguished from lymphocytes by their abundant cytoplasm and from plasma cells by a relative paucity of granular

endoplasmic reticulum. In addition macrophages were present. Our findings bear some resemblance to those reported above and are suggestive of, but do not prove a possible immunological aetiological origin.

The chromatolytic changes in the ganglion cells following guanethidine are very marked presenting a picture of foam or ghost cells when gallocyanine staining is used. MALMQUIST & OATES (1968) have demonstrated a guanethidine induced inhibition of oxidative phosphorylation in isolated rat liver mitochondria and therefore electronmicroscopic studies are being undertaken to investigate further the histological changes described above. Preliminary results have shown marked dilatation of the mitochondria of ganglion cells from rats treated with guanethidine

The histology and cholinesterase histochemistry of the superior cervical ganglion described in the present paper do not differ from the picture described by other investigators except that the staining for ChE seems to be more intense both in ganglion cells and nerve fibres, and the staining of the glial elements is less intense than usually described (see HÄRKÖNEN 1964). These differences may be due to the use of different histochemical procedures. The effects of preganglionic denervation and postganglionic axotomy on the histology cholinesterase and catecholamine histochemistry are similar to the changes described by others (HÄRKÖNEN 1964 ERÄNKÖ & HÄRKÖNEN 1965a, NAGATA & TSUKADA 1968 TAXI 1961) NAGATA & TSUKADA (1968) found an almost complete disappearance of the ganglion cells following postganglionic axotomy This is not in agreement with the present results and those described by ERÄNKÖ & HÄRKÖNEN (1965a). The latter investigators found a regeneration of the nerve cells 3 months after axotomy This difference may be explained as the result of a more complete stripping of the postganglionic nerves in the study of NAGATA & TSUKADA (1968). It seems possible that the differences in the types of small cells between the findings of these investigators and the present study is due to ischaemic changes following complete stripping of the postganglionic nerves. In the present study there was no loss of ganglion cells.

The changes induced by guanethidine in many ways resemble those resulting from postganglionic axotomy i e. morphologically cholinesterases and catecholamines as observed by histochemistry time of onset time required for a maximal effect, and lastly the partial reversibility of the changes. The basic change is assumed to be the chromatolysis, the other findings being secondary

There is a certain discrepancy between the decrease in enzymatic activity as evaluated by histochemical technique as compared to quantitative biochemical analysis. Whereas the histochemically evaluated loss is almost total 30–60 / of the control activity can still be measured by means of a titrimetric procedure (JENSEN-HOLM & JUUL 1970). This may be explained

as the result of a quantitatively inadequate histochemical technique, involving the loss of soluble esterases during staining of unfixed specimens, a partial inhibition of cholinesterases when using formaldehyde as a fixative and a possible diffusion of the end product lead-ferrocyanide. Furthermore a comparison between the histochemical and biochemical analyses is difficult, since among other things the procedures differ with regard to substrate, substrate concentration, pH, temperature, and composition of the incubation solution.

The observed disappearance of catecholamines following postganglionic axotomy is in accordance with the findings of HÄRKÖNEN (1964) and ERÄNKÖ & HÄRKÖNEN (1965a). HÄRKÖNEN (1964) however did not find the increased fluorescence following preganglionic denervation. The histochemically demonstrated depletion of noradrenaline by guanethidine corresponds well with the biochemical findings of SANAN & VOGT (1962).

The changes described in the superior cervical ganglion following guanethidine seem to be specific, as preliminary histological investigation of ganglion cells in the nodose ganglion and in the small intestine do not indicate any signs of chromatolysis. Furthermore sections of the spinal cord reveal no morphological alterations after administration of guanethidine (unpublished results). The latter finding may be due to the low concentrations of guanethidine in the central nervous system.

Although the alterations induced by guanethidine in many ways resemble those resulting from postganglionic axotomy there seem to be functional differences. Postganglionic axotomy has been shown to block ganglionic transmission (BROWN & PASCOE 1954 and others) whereas no impairment of ganglionic function could be detected following prolonged administration of guanethidine (BEIN 1960 DOWNING, JUUL & QUILLIAM unpublished). Furthermore the present investigation has shown that the changes develop irrespective of previously performed pre or postganglionic nerve division. These findings indicate a possible direct ganglionic action of guanethidine, the nature of which still remains unknown.

Acknowledgements

Some of the histological work has been performed in the Department of Neuropathology and the Department of Pathology University of Copenhagen, Denmark. The histochemical demonstration of catecholamines has been undertaken by the Department of Histology University of Lund, Sweden. Guanethidine monosulphate (Ismelin®) has been generously supplied by Danish CIBA. Most of the analyses have been performed by Miss Annette Francker This work was supported by a grant from Nordisk Insulinfond.

REFERENCES

Bein, H. J., Some pharmacological properties of guanethidine. In. *Adrenergic Mechanisms.* CIBA Foundation, (Churchill, London) 1960, 162 172.

Bodian, D A new method for staining nerve fibers and nerve endings in mounted paraffin sections. *Anat Rec* 1936, 65 89–97

Brown, G L. & J E. Pascoe The effect of degenerative section of ganglionic axons on transmission through the ganglion. *J Physiol.* (London) 1954, 123 565–573

Einarson, L., A method for progressive selective staining of Nissl and nuclear substance in nerve cells. *Amer J Path.* 1932, 8, 295–308.

Eränkö, O. & M. Härkönen: Effect of axon division on the distribution of noradrenaline and acetylcholinesterase in sympathetic neurons of the rat. *Acta physiol. scand.* 1965a, 63, 411–412.

Eränkö O. & M. Härkönen. Monoamine-containing small cells in the superior cervical ganglion of the rat and an organ composed of them. *Acta physiol. scand.* 1965b, 63, 511–512.

Eränkö O G B. Koelle & L. Räisänen A thiocholine-lead ferrocyanide method for acetylcholinesterase. *J Histochem. Cytochem.* 1967 15 674–679

Falck, B & C. Owman: A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic amines. *Acta Univ Lund.* 1965, *II* no. 7

Härkönen, M., Carboxylic esterases, oxidative enzymes and catecholamines in the superior cervical ganglion of the rat and the effect of pre- and postganglionic nerve division. *Acta physiol. scand.* 1964 63 suppl. 237

Holmstedt, B., A modification of the thiocholine method for the determination of cholinesterase. I. Biochemical evaluation of selective inhibitors. *Acta physiol. scand.* 1957 40 322–330.

Jensen-Holm, J Cholinesterase and protein changes in sympathetic ganglia after guanethidine. *Acta pharmacol. et toxicol.* 1967 25 suppl. 4 74.

Jensen-Holm, J & P Juul, Changes of the rat superior cervical ganglion induced by guanethidine (histology and cholinesterase histochemistry). *Brit J Pharmacol.* 1968, 34 211P

Jensen-Holm, J & P Juul The effects of guanethidine, pre- and postganglionic nerve division on the rat superior cervical ganglion: cholinesterases and protein. *Acta pharmacol. et toxicol.* 1970, 28, 270–282.

Karnovsky M. J & L. Roots: A direct-coloring thiocholine method for cholinesterases. *J Histochem. Cytochem.* 1964 12, 219–221

Lampert, P W Demyelination and remyelination in experimental allergic encephalomyelitis. Further electron microscopic observations. *J Neuropath. & Exper Neurol.* 1965, 24 371–385

Malmquist, J & J A. Oates: Effects of adrenergic neuron-blocking guanidine derivatives on mitochondrial metabolism. *Biochem. pharmacol.* 1968, 17 1845–1854.

Marshall, A. H. E., *An outline of the cytology and pathology of th reticular tissue* (Oliver & Boyd, Edinburgh & London) 1956).

Nagata, Y & Y Tsukada. Effect of degeneration of sympathetic ganglion cells on amino acid metabolism in cervical ganglion from the rat. *Exper Brain Res.* 1968, 5 203–209

Sanan, S. & M Vogt: Effect of drugs on the noradrenaline content of brain and peripheral tissues and its significance. *Brit J Pharmacol.* 1962, 18, 109–127

Taxi, J La distribution des cholinestérases dans divers ganglions du système nerveux autonome des vertébrés. *Bibl. anat* 1961, 2, 73–89.

Acta pharmacol. et toxicol. 1970, 28, 299–304

From the Department of Pharmacology and the Pharmacological Division of the Department of Pharmacy, University of Helsinki, Finland

A Possible Mechanism of Toxicity of Trifluoroethanol and Other Halothane Metabolites

By

Mauno M. Airaksinen, Per H. Rosenberg and Tapani Tammisto

(Received January 5, 1970)

Abstract. Several drugs modify the lethal action of trifluoroethanol (TFE) and trifluoroacetaldehyde hydrate (TFAlH). The toxic effect of these metabolites of halothane may be due to the reactions of trifluoroacetaldehyde (TFAl), since the toxicity of TFE was reduced by the administration of ethanol (500 mg/kg × 2, intraperitoneally) or 4-iodo-pyrazole (50 mg/kg × 2, intraperitoneally), an inhibitor of alcohol dehydrogenase. Furthermore, allopurinol (20 mg/kg × 2, intraperitoneally), which as an inhibitor of xanthine oxidase may decrease the further oxidation of TFAl, increased the toxicity of TFE and TFAlH but not that of trifluoroacetic acid (TFAA). Isoniazid (50 mg/kg × 2, intraperitoneally), a carbonyl reagent, effectively reduced the toxicity of TFE and TFAlH, while cysteine (500 mg/kg × 2, intraperitoneally) showed some protection against the TFE toxicity. Cysteine, *in vitro*, also prevented the inhibitory action of TFAlH and TFAA on the creatine phosphokinase (CPK) activity. TFE did not inhibit this SH-enzyme *in vitro*. Thus the formation of a thiol-binding aldehyde may be essential for the metabolic blocking effect and toxicity of TFE and TFAlH.

Key-words. Toxicology – halothane – anaesthetics.

Trifluoroacetic acid (TFAA) is the main metabolite found in the urine of man after halothane anaesthesias (Rehder *et al.* 1967). It is probably formed through trifluoroethanol (TFE) and trifluoroacetaldehyde (TFAl) (Van Dyke & Chenoweth 1965), though another route has also been suggested (Stier 1967).

The individual toxicities of the metabolites are seen in table 1 (Airaksinen & Tammisto 1968). TFE has been shown to lower the ATP/ADP ratio in mouse liver more than the creatine phosphate/creatine ratio in mouse muscle. This discrepancy was thought to be due to a partial inhibition of creatine kinase (CPK). Halothane metabolites also inhibited anaerobic glycolysis (Rosenberg *et al.* 1970).

Table 1

Modification of the toxicity of TFE, TFAIH and TFAA in mice by some drugs injected intraperitoneally 10 min. before and 3 hrs after the trifluoro-compound.

Treatment	LD50 ± S. E. M. mg/kg i. p.		
	TFE	TFAIH	TFAA
None	195 ± 15	650 ± 65	> 2000
L-cysteine 500 mg/kg × 2	265 ± 17	795 ± 35	> 2000
Isoniazid 50 mg/kg × 2	390 ± 33	1060 ± 80	> 2000
Ethanol 500 mg/kg × 2	485 ± 38	700 ± 55	> 2000
4-Iodo-pyrazole 50 mg/kg × 2	450 ± 40	680 ± 35	> 2000
Allopurinol 20 mg/kg × 2	63 ± 9	45 ± 8	> 2000

TFE and TFAl hydrate (TFAIH) seem to be hepatotoxic in mice, causing fat accumulation in the liver within 24 hrs after single non-lethal doses (ROSENBERG & WAHLSTRÖM 1970).

The present study was undertaken in order to evaluate the probable mechanism of the toxicity of halothane metabolites, with special reference to the binding of active thiol groups by trifluoroacetaldehyde.

Material and Methods

Toxicological study

The drugs, trifluoroethanol (TFE, Fluka AG Buchs SG), trifluoroacetaldehyde hydrate (TFAIH, Pierce Chem. Co., Rockford, Illinois) trifluoroacetic acid sodium (TFAA, Schuchardt, München) L-cysteine (Fluka EG Buchs SG), ethanol (OY Alko AB, Helsinki), isoniazid (Lääketehdas Orion OY Helsinki) 4-iodo-pyrazole (a gift from Dr B. Sjöberg, Astra Ltd. Södertälje) and allopurinol (OY Medica AB, Helsinki) were dissolved in saline and injected intraperitoneally into male Swiss albino mice (17–20 g).

The deaths among the mice during three days were counted, the mean lifetime of those who died after LD50–LD90 dose was approximately evaluated, and the LD50 (+ S. E. M.) was estimated using the graphic method of MILLER & TAINTER (1944). At least four groups each of 10 mice were used for each LD50 determination.

In vitro study of thiol-binding property

TFE, TFAIH and TFAA were preincubated with creatine phosphokinase (CPK, Boehringer Mannheim), an enzyme with SH groups essential for its action.

The CPK activity was measured at 25° by a modified method of TANZER & GILVARG (1959), using the reagent kits of Boehringer (Mannheim). The reagents also included two enzymes with active SH groups: lactic dehydrogenase (LDH) and pyruvate kinase (PK).

L-cysteine, which is known to reverse the SH-inhibitions of different enzymes (MADSEN 1963) was added to the test medium in equimolar amounts together with the test drug.

Results

Modification of the toxicity

Table 1 presents the effect of ethanol and some metabolic inhibitors on the toxicity of TFE, TFAIH and TFAA. Ethanol in a dose of 500 mg/kg × 2 protected against the toxicity of TFE.

Isoniazid was the most effective protector against TFE and TFAIH toxicity. 4-Iodo-pyrazole also protected fairly well against the toxicity of TFE but not against that of TFAIH. Cysteine slightly decreased the toxicity of TFE but not that of TFAIH.

Allopurinol in a high dose, markedly enhanced the toxicity of both TFE and TFAIH, but did not modify the toxicity of TFAA.

The effect of TFE and TFAIH on the histological picture and weight of the mice liver will be published separately (ROSENBERG & WAHLSTRÖM 1970).

Effect on enzymes with active thiol groups

TFAA and TFAIH inhibited the CPK activity (together with the LDH and PK activities) *in vitro* (fig. 1a). The inhibition was dose-dependent and at all concentrations was more marked with TFAA than with TFAIH.

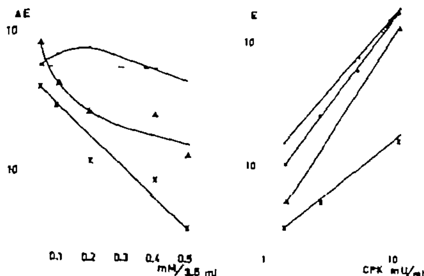


Fig. 1a & b. Inhibition of CPK activity *in vitro* by TFE ( ), TFAIH (▲) and TFAA (×).

a) The effect of inhibitor concentration (half logarithmic scale). The extinction (ΔE) without any inhibitor is marked with a broken line.

b) The effect of enzyme concentration (logarithmic scale). CPK controls are marked with large dots (●).

Table 2

Inhibition of CPK activity *in vitro* by halothane metabolites. The concentrations of the inhibitors as well as of cysteine were 0.2 mmoles/3.5 ml. The values are the means of 3 determinations.

	Inhibition %	
		With cysteine
TFE	− 18.3	0
TFAlH	52.5	14.7
TFAA	88.7	− 7.3

The inhibition caused by TFAlH was prevented when the activity of CPK was increased (fig. 1b), but the inhibition by TFAA tended to increase as the enzyme activity increased.

Cysteine totally reversed the inhibition caused by TFAA and almost totally that caused by TFAlH (table 2)

Discussion

Since hydrazides are known to be carbonyl reagents (CLARK 1963) isoniazid and other hydrazides are probably bound to trifluoroacetaldehyde and thus protect against its toxic actions. The findings support this assumption since both the lethal and the liver effects (ROSENBERG & WAHLSTRÖM 1970) were antagonized.

In the case of the protection given by cysteine, the mechanism might depend on an *in vivo* activation of different thiol compounds, resulting in a reversal of an anticipated thiolester formation (SCHUBERT 1936) by trifluoroacetaldehyde and SH-groups of enzymes.

TFE has been found to be a competitive inhibitor of alcohol oxidation, but TFE did not inhibit liver or yeast alcohol dehydrogenase (ADH) *in vitro* (BLAKE *et al.* 1969). ADH however seems, to be involved in the step from TFE to TFAl, since TFAlH was slowly reduced by mammalian ADH to TFE (OTANI personal communication) TFE has, indeed, been recently identified as a metabolite of TFAlH in rats by gas chromatographic analysis of blood (AIRAKSINEN *et al.* unpublished)

4-iodo-pyrazole inhibits ADH (RYDBERG 1969) and therefore probably prevents the formation of TFAl from TFE. TFE was shown to be without any effect *in vitro* on fibroblasts lacking ADH, whereas TFAlH inhibited their growth (ROSENBERG & WAHLSTRÖM 1970)

The protective effect of ethanol on the toxicity of TFE, as also shown by BLAKE *et al* (1969), seems to depend on the greater affinity of ethanol to ADH, which in turn prevents the metabolism of TFE.

Xanthine oxidase seems to catalyze the reaction from TFAl to TFAA, since allopurinol, a xanthine oxidase inhibitor (RUNDLES *et al.* 1963), potentiated the toxicity of both TFE and TFAlH but not that of TFAA. The opposite effect of allopurinol on TFE reported by BLAKE *et al.* (1969) may have been due to a different time of administration (4.5 hrs before TFE)

The inhibition of CPK activity *in vitro* seems to be due to a SH inhibition, since the inhibition afforded by TFAlH and TFAA was reversed in both cases by cysteine. The inhibition by these two metabolites was not quite similar since the inhibition by TFAA was increased by increasing the CPK activity This probably indicates that the action of some of the cofactors or reagents, e. g. LDH and PK, was partially inhibited.

Although TFE has been shown to be the most toxic of the halothane metabolites *in vivo* (AIRAKSINEN & TAMMISTO 1968 BLAKE *et al.* 1969 TFE itself did not inhibit SH-groups *in vitro*

The formation of a SH inhibitor is further suggested by the decrease in the concentration of reduced glutathione in mice liver and erythrocytes by TFE and TFAlH *in vivo* but not *in vitro* (ROSENBERG unpublished)

It is tentatively concluded from these studies that trifluoroacetaldehyde, or a compound derived from it, may be responsible for the toxic actions of TFE. TFAl may be formed from halothane either directly or through TFE. Though TFAA is also formed, the effect of allopurinol seems to argue against its role *in vivo*

Acknowledgements

The study was supported in part by grants from Alkoholitutkimussäätiö (The Finnish Foundation for Alcohol Studies) and Valtion luonnontieteellinen toimikunta (The National Research Council for Natural Sciences in Finland) Our thanks are due to Mrs. Katarina Mäkynen and Mrs. Ritva Aarnuharju for technical assistance.

REFERENCES

Airaksinen, M. M & T Tammisto: Toxic actions of the metabolites of halothane: LD50 and some metabolic effects of trifluoroethanol and trifluoroacetic acid in mice and guinea pigs. *Ann. Med. exp Fenn.* 1968, 46, 242 248.

Blake, D. A., H. F Cascorbi, R. S. Rozman & F J Meyer Animal toxicity of 2,2,2 trifluoroethanol. *Toxicol. Appl. Pharmacol.* 1969 15, 83-91.

Clark, W G. Inhibition of amino acid decarboxylases. In. *Metabolic inhibitors. A comprehensive treatise* Vol. I. Ed. R. M Hochster & J H. Quastel. Acad. Press, New York & London 1963.

Madsen, N B Mercaptide forming agents. In: *Metabolic inhibitors. A comprehensive treatise* Vol. II. Ed. R. M Hochster & J M. Quastel. Acad. Press, New York & London 1963

Miller L. C. & M L. Tainter: Estimation of the ED50 and its error by means of logarithmic probit graph paper *Proc Soc exp Biol.* (N Y.) 1944 57 261 264.

Rehder K., J Forbes & H Alter: Halothane biotransformation in man. A quantitative study *Anesthesiology* 1967 28 711 715

Rosenberg, Per H Mauno M. Airaksinen & Tapani Tammisto: Inhibition of energy production by halothane metabolites. *Acta pharmacol. et toxicol.* 1970, 28, in press.

Rosenberg, Per H & Torsten Wahlström. Hepatotoxicity of halothane metabolites *in vivo* and inhibition of fibroblast growth *in vitro Acta pharmacol. et* toxicol. 1970, 28 in press.

Rundles, R. W J B Wyngaarden, G H. Hitchings, G B. Elion & S. Silberman. Effects of a xanthine oxidase inhibitor on thiopurine metabolism, hyperuricemia and gout. *Trans. Ass. Amer Phy iol.* 1963 76, 126–140.

Rydberg, U S. Inhibition of ethanol metabolism in vivo by 4-iodo-pyrazole. *Biochem. Pharmacol.* 1969 18, 2425–2428.

Schubert, M. P Compounds of thiol acids with aldehydes. *J Biol. Chem.* 1936, 114 341 350.

Stier A The biotransformation of halothane. *Anesthesiology* 1967 29 388–389.

Tanzer M. L. & C. Gilvarg: Creatine and creatine kinase measurement. *J Biol. Chem.* 1959 234 3201–3204

Van Dyke, R. A. & M. B Chenoweth: Metabolism of volatile anesthetics. *Anesthesiol gy* 1965 26, 348–357

Acta pharmacol. et toxicol. 1970 28 305–326.

From the Pharmacology Section, Division of Experimental Defence Medicine,
Research Institute of National Defence, Department 1,
Stockholm, Sweden

Central and Peripheral Effects of Anticholinergic Compounds

By

Lennart Albanus

(Received February 24 1970)

Abstract. The pharmacological effects of aryl and cycloalkylaryl glycolates of piperidin, tropine and quinuclidin have been studied in relation to atropine and scopolamine. Peripheral effects (heart rate, salivation, pupil size) have been studied in dogs in relation to their behavioural effects under standardized conditions. The anticholinergic activity was further investigated on the mouse pupil and on the blood-pressure response to acetylcholine in cats anaesthetized with pentobarbital. In dogs all the glycolates elicited behavioural effects, especially on locomotion, similar to those seen following atropine and scopolamine. Even high doses of methyl atropine produced effects indistinguishable from those produced by atropine and scopolamine. The most active compounds affected behaviour at a dose of 10 µg per kilogram body weight given subcutaneously. All compounds elicited anticholinergic activity i.e. tachycardia, mydriasis, inhibition of salivation and block of AcCh response. The most potent compounds on behaviour were quinuclidinylesters of phenyl and/or thienyl glycolic acid which also had the strongest and most prolonged classical anticholinergic effects. The results indicate that behavioural effects are related to anticholinergic activity but that other factors such as lipid-solubility and metabolism interfere with the central activity.

Key-words: Parasympatholytics – glycolates – behaviour animal – belladonna.

Large doses of atropine and scopolamine elicit effects on the central nervous system which are manifested by impaired memory restlessness, ataxia, hallucinations, delirium and coma. In dogs a bizarre behaviour has been reported following both atropine and scopolamine (White *et al.* 1961). Similar effects are also produced by some highly potent anticholinergic glycolates (Abood & Biel 1962 Bell *et al.* 1964 Bell & Gershon 1964 Gershon 1966 Abood 1968). The glycolates, as well as LSD have been claimed to be potential psychochemical warfare agents (Franke 1967)

In the present paper the pharmacological effects are described of some piperidyl tropinyl and quinuclidinylesters of glycolic acid as compared with atropine scopolamine and methyl atropine. Their peripheral anticholinergic effects have also been studied in relation to their effects on behaviour in the dog.

Methods

Central and peripheral effects in dogs.

Fifty dogs, adult beagles of both sexes, from our own kennel were used. Even before the tart of the experiments, they were accustomed to the experimental environment and procedure during at least 20 hours. The experimental room was divided in two compartments (2.4 × 2.4 meters) by a wirenetting. In each of the compartments, a horizontal ladder was fastened 20 cm from the floor and reaching one metre into the room in order to study the dogs ability to avoid obstacles. The behaviour of the dogs was *observed from an adjacent room through a one-way-mirror. The experimental* room was semi-soundproof and artificially lighted. The temperature was kept at 20–24 nd the relative humidity varied between 40–60 per cent. The dogs were given repeated injections (about four) but at least two weeks were allowed to elapse between the injections.

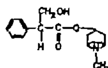
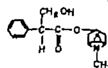
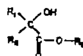
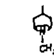
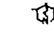
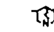
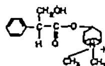
The experiments were carefully standardized and the procedure was as follows: Two dogs were fed and left alone in the experimental room for one hour. The observer then entered the room and made a standard control investigation (see below). The behaviour of the dogs was observed from the adjacent room for the next 45 min., after which control number two was taken and the test drug or saline injected subcutaneously into the back of the neck. Every 45 min. the same investigation was performed and in intervening periods the behaviour of the dogs was continuously observed and recorded on video tape for about 1 min. every fifteen min. and used for further analysis. The duration of the experiment was bout five hours. The observer was not informed of the drug or dose injected.

Assessment f symptoms.

The behaviour during the 45 min. periods was assessed by means of a rating scale. Spontaneous activity balance, taxia, tremor vomiting, spontaneous utterance of sound, blundering into objects, degree of alertness and reaction to sharp noise were recorded. Stiff gait, stumbling and staggering were regarded as different degrees of ataxia. The blundering of the dogs into objects was considered to be of first degree when the animals walked into objects but retreated, and of second degree when the dogs did not withdraw but attempted t "push through the objects. The spontaneous activity was measured by recording as period of time in motion, standing or sitting still and recumbent, respectively

During the control periods when the observer entered the room, certain autonomic parameters were measured. heart rate was recorded by auscultation and the pupil size was measured by more exact description hole-slice. The reaction of the pupil to light was assessed by directing a standardized light into the eye. The observer also estimated activity response of the dogs as well as their attitude towards the observer and reaction to sharp noise.

Fig. 1. Name, formula, molecular weight and code numbers of compounds investigated.

Code number	Name and molecular weight	Formula		
1	atropine, 289.4			
2	scopolamine, 303.4			
				
		R_1	R_2	R_3
3	Ditran JB-329 328.0			
4	3-tropyl benzilate, 351.4			
5	3-tropyl-9-hydroxy-9-fluorene carboxylate, 349.4			
6	3-quinuclidinyl-9-hydroxy-9-fluorene carboxylate, 335.4			
7	1-methyl-4-piperidyl benzilate, 325.4			
8	3-quinuclidinyl benzilate, Ro2 3308, QB, 337.4			
9	3-quinuclidinyl-cyclopentylphenyl glycolate, 329.4			
10	3-quinuclidinyl-2-thienyl-phenyl glycolate, 343.4			
11	3-quinuclidinyl 2,2'-thenilate, 349.2			
12	3-quinuclidinyl 3,3'-thenilate 349.2			
13	methyl atropine, 304.4			

The degree of salivation was estimated by inspecting the mucous membrane of the overlip and was graded into three classes.

The four observers were trained by means of demonstrations to assess the different symptoms and a good correlation was obtained when the records of two independent observers were compared.

Mydriatic effects on mice

The mydriatic effect was studied in mice, CBA, males, 24–27 g body weight, according to PULEWKA (1932) using a microscope with a graded ocular and standardized light (Bausch and Lomb, BCB 73). The drugs were given intraperitoneally or by local application to groups of 5 animals. When the drugs were given as eyedrops, the pH was adjusted to about 7 The pupil size was determined at least hourly for four hours or longer

Blocking effect on blood-pressure response to acetylcholine

Cats, 2.9 kg (4.2 1.7), 4 ♂ 29 ♀ were anaesthetized with pentobarbital sodium (mebumalum NFN) and the carotic blood pressure was recorded by a transducer (Statham P23BC) linked to a recorder (Grass Model 5 Polygraph). The depth of anaesthesia was controlled by repeated small doses of the anaesthetic. Drug injections were made via a standardized catheter into one femoral vein. The blood pressure response to 0.25, 0.50 and 0.75 μg acetylcholine hydrochloride (AcCh) per kilogram body weight was first determined. After this the cholinolytic drug was injected and the response to AcCh tested after 10, 20, 30 and 60 min. The order in which the AcCh injections were given was randomized.

Substances.

The compounds investigated are listed in fig. 1 in which the formulae, molecular weights and code numbers are given. Atropine sulphate, scopolamine hydrobromide and methyl atropine nitrate (compounds 1 2 and 13) were commercial products. Ditran® (JB 329), compound 3, is a mixture of N-ethyl-2-pyrrolidyl-methyl cyclopentyl-phenyl glycolate (70 %) and N-ethyl-3-piperidyl cyclopentyl-phenyl glycolate (30 %) which was kindly supplied by Lakeside Laboratories, Milwaukee, Wisconsin. Compounds 4 to 12 were synthetized in the Division of Applied Organic Chemistry at the Research Institute of National Defence, Stockholm, compounds 4 to 10 by FLORMARK & LARSSON (unpublished) and compounds 11 and 12 by NYBERG et al. (1970) and used as hydrochlorides.

Results

Effects on behaviour

The effects of atropine, scopolamine, methyl atropine and the glycolates on the behaviour of the dog were studied following the subcutaneous injection of 0 005 to 20 mg of the salts per kilogram body weight. A summary of the main effects and their time course is given in tables 1 2 and 3 The number of the compounds refers to fig. 1

All the compounds elicited a very similar behaviour pattern. Therefore, only the effects of atropine (0.5 mg/kg subcutaneously) will be described in

Table 1

Time-course of ataxia and non-retreating behaviour

Compound	Dose s. c. mg/kg	Behaviour min. after injection: Ataxia — Appearance	Ataxia — Disappearance	Non-retreating — Appearance	Non-retreating — Disappearance	Number of experiments
1	0.10	0	0	0	0	4
	0.20	0/1 65/3	0/1 158/3	0	0	4
	0.30	68	264	0/2 110/3	0/2 157/3	5
	0.50	39	279	68	231	7
	2.50	16	> 315	31	290/1 > 315/3	4
2	0.01	0	0	0	0	2
	0.03	53	223	0	0	6
	0.05	32	217	65	133	4
	0.50	11	290/2 > 315/2	16	193	4
3	0.05	0	0	0	0	2
	0.50	22	275/10 > 315/1	31	231	11
4	0.05	0/2 57/2	0/2 141/2	0	0	4
	0.50	16	275/4 > 315/1	19	244	5
5	0.05	0	0	0	0	2
	0.50	31	237	56	198	3
6	0.05	0	0	0	0	4
	0.50	50	239	41	215	3
7	0.05	29	146	64	126	4
	0.50	16	270	34	210	2
8	0.01	113	> 315	0/1 150/1	0/1 > 315/1	2
	0.05	36	295/1 > 315/11	42	285/4 > 315/8	12
9	0.01	0/2 74/4	0/2 196/4	0	0	6
	0.05	29	178	47	149	8
	0.50	13	> 315	16	> 315	2

Compound	Dose s. c. mg/kg	Behaviour min. after injection: Ataxia – Appearance	Ataxia – Disappearance	Non-retreating – Appearance	Non-retreating – Disappearance	Number of experiments
10	0 005	86	193	0	0	4
	0 01	75	> 315	140	> 315	4
	0.05	19	> 315	27	> 315	2
11	0 01	50	241/3 > 315/5	0/2 130/6	0/2 230/4 > 315/2	8
	0.05	18	> 315	24	276/3 > 315/1	4
	0.55	9	> 315	17	> 315	2
12	0.01	0/2 125/4	0/2 300/2 > 315/2	0/5 112/1	0/5 291/1	6
	0 02	120	268/1 > 315/1	0/1 145/1	0/1 215/1	2
	0 03	82	268/1 > 315/3	0/2 138/2	0/2 208/2	4
	0.05	25	> 315	50	> 315	4
13	1.00	0	0	0	0	4
	2.50	0	0	0	0	4
	10.00	0/3 85/5	0/3 270/5	0/5 120/3	0/5 240/3	8
	20.00	72	295/2 > 315/2	84	265/2 > 315/2	4

/4 = number of observations.

detail. The first effects noted were those affecting locomotion (40 min. after injection) The gait lost its normal elasticity and the hindlegs became stiff (ataxia) In particularly lively animals, the movements became slow Some what later small quick ear movements and sudden head jerks became apparent. The dogs sometimes started chewing and stroked their nose with one paw or against a foreleg. The eyes were half closed. Often the dogs became surprisingly interested in details of their surroundings, for example a spot on the floor They could also be seen trying to catch invisible flies or lifting their legs high as if they were stepping over an invisible obstacle. There was also a change in the dogs reactions to certain external stimuli. Thus, they did not respond by increased motor activity when the observer visited them nor did they approach the visitor (in their normal manner) Their reaction

Table 2

Effects of the compounds on the dogs attitude to the environment judged from their response to the observer

Compound no.	Dose mg/kg	Time after injection (min.) 45	90	135	180	225	270	315
1	0.50	∓	—	—	—	—	∓	∓
2	0.05	∓	∓	+	∓	∓	∓	∓
3	0.50	—	—	—	—	—	∓	∓
4	0.50	—	—	—	—	—	—	—
5	0.50	∓	∓	—	∓	∓	∓	∓
6	0.50	—	—	—	—	—	∓	+
7	0.05	∓	∓	+	+	+	+	+
8	0.01	+	+	+	∓	∓	∓	∓
	0.05	—	—	—	—	—	—	∓
9	0.05	—	—	—	+	+	+	+
10	0.01	∓	∓	—	—	—	—	∓
11	0.01	—	—	—	∓	—	∓	∓
12	0.01	+	∓	∓	∓	+	+	+
13	10.00	+	∓	∓	∓	∓	+	+
	20.00	+	∓	—	—	—	—	∓

Symbols: — loss of contact
∓ decreased contact
\+ normal

to sharp noises (for example a strong hand clapping) was decreased and the ability to localize a whistle signal was impaired. They turned their heads from side to side, often with the nose pointing to the floor unable to localize the signal. At the height of the intoxication, locomotion was characterized by a stumbling gait, impaired balance and weakness in the hind legs and the dogs blundered into objects. They bumped their nose against the netting or the walls or knocked their forelegs against the ladder At first the dogs immediately withdrew but later on, about 70 min. after the injection, they no longer retreated but tried to push through the object no matter if it was a wall or the ladder (non-retreating, table 1) They often pushed their heads into a corner or became intangled in the horizontal ladder making awkward attempts to force the obstacle. They took no notice when they walked into their water bowl. Sometimes the animals were seen stumbling along the walls on flexed forelegs and lowered back and with the hindlegs placed more forward and under the body than is normally seen. When standing, they swayed from side to side, or also backwards and forwards which caused the dogs to start walking. Their motor activity was generally increased. At this point, the dogs often barked or whined and sometimes they panted.

Table 3

Effects on salivation and light reflex of the eye at different times following administration of the compounds in doses giving behavioural effects.

Compound no.	Dose mg/kg	Time after injection (min.) 45	90	135	180	225	270	315
1	0.50	− ○	− ○	− ○	∓ ○	+ ○	+ ○	+ ○
2	0.05	− ○	− ○	∓ ○	∓ ○	∓ ○	+ ○	+ ○
3	0.50	∓	− ⊙	− ○	∓ ○	∓ ○	∓ ⊙	∓ ⊙
4	0.50	− ○	− ○	− ○	− ○	∓ ○	∓ ○	∓ ○
5	0.50	− ○	− ○	− ○	∓ ⊙	∓ ○	∓ ○	∓ ⊙
6	0.50	∓ ○	∓ ○	− ○	− ○	∓ ○	∓ ○	∓ ○
7	0.05	−	−	−	−	−	∓ ⊙	∓ ⊙
8	0.01	∓	∓	∓ ⊙	− ○	− ○	− ⊙	∓ ⊙
	0.05	∓	− ○	− ○	− ○	− ○	− ○	− ○
9	0.05	∓	−	∓	∓	∓	∓	∓
10	0.01	∓	∓	∓	−	−	∓	−
11	0.01	−	− ⊙	− ○	− ⊙	− ○	− ○	∓ ○
12	0.01	∓	∓		−	−	−	−
13	10.00	− ○	− ○	− ○	− ○	− ○	− ○	− ○
	20.00	− ○	− ○	− ○	− ○	− ○	− ○	− ○

Symbols: Salivation. − decreased Light reflex. ○ no
∓ normal ⊙ slow
\+ increased normal

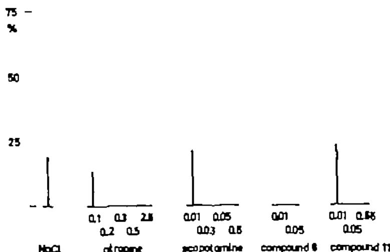


Fig. 2. Motor activity in dogs. Per cent of time spent for muscl activity during 315 min. following injection (mg/kg).

All the dogs vomited one to four times (mainly 45 to 90 min. after the injection) The symptoms of non-retreating began to disappear after 200 min. but the ataxia was seen until about 5 hours after the injection. The next day their behaviour was normal.

A higher dose of atropine (2.5 mg/kg atropine sulphate) was given to four dogs. Essentially the same effects were noticed but the onset of the symptoms was shorter 10 to 20 min. after injection the dogs manifested ataxia and walked into objects, and between 25 to 35 min. after treatment they no longer retreated. These symptoms, including an absence of reaction to the entrance of the observer lasted about five hours but after 22 hours no impairment of behaviour was observed.

In another series of experiments, the threshold dose was studied. After 0.1 mg/kg to four dogs, no behavioural effects could be detected. After 0.2 mg/kg to another group of four dogs, some degree of ataxia was observed from one to two and a half hours after the injection in three of the dogs. Two animals vomited one to one and a half hours after the drug was given. All the dogs also showed an increased spontaneous motor activity (fig. 2) No other effects on gross behaviour could be detected. Doses of 0.3 mg/kg given to five dogs caused ataxia in all the animals from 68 to 264 min. after injection. Three dogs blundered into objects in their vicinity without retreating. This was observed 110 to 157 min after the injection. The spontaneous motor activity of the dogs was increased. Thus, after 0.3 mg/kg of atropine sulphate, the period during which motor activity could be noted was about 40 per cent longer than after 0.2 mg/kg The (conditioned) increase in motor

activity seen when the observer visited the dogs was decreased from 90 to 315 minutes after the injection All the dogs vomited one to four times during 30 to 90 min after the injection. One dog showed decreased reaction to sharp noise and could only localize a whistle signal with difficulty three to three and a half hours after the injection. Another dog reacted differently and reacted strongly to sharp noises.

In similar experiments with scopolamine hydrobromide, the behavioural effects were elicited at doses that were about ten times lower than for atropine sulphate. No effect on behaviour was observed following 0 01 mg/kg of scopolamine With 0 03 mg/kg the stiff gait appeared after about 45 to 75 min. in all dogs and was observed for about four hours. Two out of six dogs vomited within 40 to 70 min. after the drug was given All dogs were hyper sensitive to sharp noise after 90 min. A dose of 0 05 mg/kg caused ataxia in all the dogs within 45 min and this persisted for three and a half hours. The non-retreating behaviour started after about 60 min. and lasted about one hour Two dogs vomited at the time when the ataxia started.

In summary atropine and scopolamine elicit gross behavioural disturbances which, in lower doses or at the commencement of the symptoms following high doses, are manifested as ataxia. If the dose given is high enough blundering against objects is also noted. In the early phase of this syndrome the dogs retreat from the obstacle but later on a non-retreating behaviour appears together with other signs of loss of contact with the environment.

Following this detailed description of the behavioural effects caused by different doses of atropine and scopolamine, a shorter description will be given concerning the effects of the glycolates (compounds 3–12) All the drugs listed in fig. 1 evoked the same pattern of disturbances and it was impossible for the observers to distinguish between the different drugs by mere observation of the symptoms. However there were differences in the time-course and potency between the compounds.

Table 1 summarizes the time-course of ataxia and non-retreating behaviour following different doses of the compounds. From the behavioural effects of the glycolates, these can be divided into three groups on the basis of potency and time-course of the effects.

The most active drugs – comprising group one – are quinuclidinylesters of diphenyl phenylthienyl and 2-dithienyl- and 3-dithienyl-glycolic acid (compound 8, 10 11 and 12) All these drugs produced ataxia following the administration of 0 01 mg/kg. Non retreating behaviour was observed in half the number of dogs following 0 01 to 0.02 mg/kg. With a dose of 0 05 mg/kg marked effects on behaviour were observed, similar to those produced by 2.5 mg/kg of atropine.

Group number two with about the same potency but of shorter duration,

consists of scopolamine, piperidyl benzilate and quinuclidinyl phenyl-cyclopentyl glycolate (compound 2, 7 and 9) These drugs induce a fully developed syndrome following 0 05 mg/kg but the effects disappear after about 180 min. Following compound 7 ataxia and non-retreating lasted half an hour less than compound 9 Scopolamine has an intermediate duration for non-retreating.

Group number three consisted of the less active drugs i.e. atropine, Ditran® tropyl benzilate, tropyl- and quinuclidinyl-hydroxyfluorene carboxylate (compound 1 3 4 5 and 6). These drugs given in a dose of 0 05 mg/kg do not cause the central anticholinergic syndrome although ataxia was observed after substance 4 When the doses were increased ten times, all the substances in this group caused ataxia and non retreating, with time-courses similar to those seen following the same dose of atropine.

From table 1 it can be seen that following all the compounds, an increase in the dose led to a more rapid onset of ataxia and non retreating behaviour and also to a prolongation of these effects Ataxia was always seen before the non-retreating behaviour but when the dose was increased the time-lag between these symptoms became shorter When a sub-limit dose for non retreating was given, only ataxia was observed.

A decreased contact with the environment, judged from the dogs behaviour when the observer visited them, was always seen following doses which elicited the non-retreating syndrome (table 2). An increase in motor activity was seen following the administration of all compounds (fig. 2). Vomiting occurred following all the compounds except piperidyl benzilate.

In order to evaluate the importance of the peripheral effects *per se* for the disturbed behaviour the effect of methyl atropine was tested. Neither 1 mg/kg (3 dogs) nor 2.5 mg/kg (4 dogs) of methyl atropine caused any sign of the behaviour syndrome described above although the peripheral effects were pronounced. However when the dose of methyl atropine was increased to 10 mg/kg the dogs showed the same disturbed behaviour as seen following a 35 times lower dose of atropine. 20 mg/kg of methyl atropine elicited the behaviour syndrome in all the dogs for more than three hours (table 1).

As mentioned earlier saline was used as placebo All records pertaining to the placebo trials were negative with regard to the behavioural effects described above.

Effects on heart rate pupil size salivation and AcCh response.

The heart rate was measured on two occasions before injection (see methods) and was found to be rather constant 106.1 ± 1.2 beats/min. (mean and S.E.M. from 145 experiments). The effects of the drugs were measured at 45 min. intervals and are expressed in per cent of the basal values. In the control experiments with saline the heart rate was somewhat decreased dur

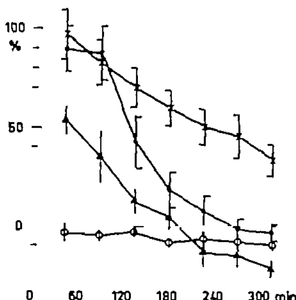


Fig. 3. Heart rate following compound 11 0.01 mg/kg (X), scopolamine: 0.05 mg/kg (▲), atropine: 0.5 mg/kg (●), and saline: 0.1 ml/kg (○). Ordinate: % increase of basal rate. Vertical bars: standard error of the mean.

ing the experiments (see fig. 3). Maximum effects following the compounds were recorded after 45 min. (first measurement) except after very low doses, when maximum effects were sometimes obtained at 90 min. The time course of the effects of atropine, scopolamine and compound 11 is shown in fig. 3. Following compound 11 (0.01 mg/kg) the heart rate increases about 100 % above the basal rate and is still markedly increased after 315 min. (33 %). Scopolamine given in a 5 times higher dose elicits about a 60 % increase and the duration is only about 150 min. Atropine in a dose of 0.5 mg/kg (10 times higher than that of scopolamine) gives a maximum heart rate similar to compound 11 (90 %) but the duration of the effect is much shorter.

The dose response relationship of atropine and compound 10 is shown in table 4. Effects on the heart rate and behaviour are both shown. Following a low dose (0.1 mg/kg) of atropine, a 60 % increase in the heart rate occurred without any signs of behavioural effects. Doubling this dose caused a heart rate increase of 75 % and elicited slight ataxia. Further doubling of the dose (0.5 mg/kg) produced a tachycardia in 90 % and also marked behavioural impairment. Compound 10, on the other hand, gave a maximum increase in heart rate of only 20 % when slight ataxia was produced. At doses eliciting the full behavioural syndrome, the maximum heart rate was 96 % above the basal rate. A similar relationship between heart rate and behaviour was also evident following compound 2 and 9 (table 5). Methyl atropine in a dose of

Table 4

Dose response relationship of atropine and compound 10 on heart rate and behaviour

Behaviour group	Compound	Dose mg/kg	Maximal heart rate*	Behaviour
III	1 (atropine)	0.1	61	no ataxia
		0.2	76	slight ataxia in some dogs
		0.3	76	ataxia in all dogs, non-retreating in some
		0.5	89	ataxia and non-retreating
		2.5	110	ataxia and non-retreating
I	10	0.005	20	slight ataxia
		0.01	96	ataxia and non-retreating
		0.05	147	ataxia and non-retreating

* Increase in per cent of basal rate.

1 mg/kg gave an increase in heart rate of 70 % and about the same maximum increase was obtained following 2.5 10 and 20 mg/kg but the duration of the tachycardia was prolonged when the dose was increased. Behavioural effects were seen only following high doses (10 and 20 mg/kg)

The relationship between the effects on behaviour and the heart rate is further shown in table 6 With the threshold dose for non retreating be haviour the compounds forming group one, elicit almost the same maximum increase in heart rate. The compounds of group three have similar effects on the heart rate For the compounds of group two, on the other hand, the maximum increases differ markedly Since the effects in this group on both behaviour and heart rate are very shortlasting and the first measurement is performed after 45 min. it is conceivable that the maximum effects appeared

Table 5

Maximal tachycardia at threshold dose slight ataxia.

Behaviour group	Compound	Dose mg/kg	Heart rate % increase
I	10	0.005	20
II	2	0.03	24
	9	0.01	31
III	1	0.2	76
	4	0.05	94

Table 6

Duration of the non-retreating behaviour and heart rate increase t threshold dose for behavioural effects.

Behaviour group	Compound	Heart rate* Maximal	Heart rate* after 315'	Duration of non-retreating behaviour*
I	8	80	2	290 – > 315
	10	96	49	> 315
	11	97	33	230 – > 315
	12	98	31	291
II	2	59	–22	133
	7	31	– 6	126
	9	70	11	149
III	1	89	3	231
	3	99	27	231
	4	86	38	244
	5	115	6	198
	6	75	– 4	215

* per cent increase of normal.
min. after injection.

Table 7

Duration of tachycardia and behavioural disturbances.

Dose mg/kg	Behaviour group	Compound	Heart rate % increase 315 min. after injection	Duration* ataxia	Duration* non-retreating
0.05	I	8	28	> 315	285 – > 315
		10	66	> 315	> 315
		11	56	> 315	276 > 315
		12	73	> 315	> 315
	II	2	–22	217	133
		7	– 6	146	126
		9	11	179	149
0.50	II	2	–15	290 – > 315	193
		7	16	270	210
		9	69	> 315	> 315
	III	1	3	279	231
		3	27	275	231
		4	38	275 – > 315	244
		5	6	237	198
		6	– 4	239	215

min. after injection.

before the first measurement was taken. With the doses shown in table 6 most of the dogs showed behavioural effects but even those that did not show any disturbances in behaviour nevertheless had the same degree of tachycardia following the same compound.

At the end of the experiments (after 315 min.) a 30–50 % increase in heart rate was evident following the compounds of group one, except for number 8 Following treatment group two, no tachycardia was observed (table 6). The longer duration of the tachycardia following the compounds of group one is evident when compared with group two given the same dose (0 05 mg/kg) As seen in table 7 the heart rate in group one was increased from 28 to 73 / while in group two this was not above 11 / Compound 9 had the most prolonged effect in the latter group, as is further evident when the dose was increased to 0.5 mg/kg. The table also shows the durations of both ataxia and non-retreating. It is seen that a prolonged duration of these effects is accompanied by a long duration of the tachycardia.

Salivation was depressed following all compounds (table 3). The in dividual variation in response was rather large and in some dogs an increased salivation was seen following this depression. With threshold doses for be havioural effects, atropine, scopolamine and ditran® had a rather short ac tion, while with compounds 7 11 12 and methyl atropine (13) the effect lasted for several hours.

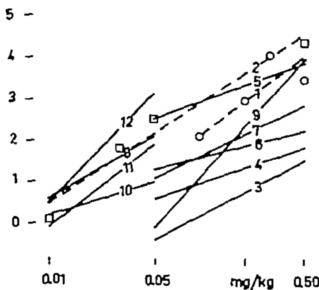


Fig. 4. Mydriatic effects in dogs. M ximal response within 315 min. in scale units (abscissa). Full lines connect results t two doses. Dotted lines drawn according to esults following several doses of compound 1 (○) and 2 (□). Numbers refer to compounds in fig. 1.

The effects on the pupil size are expressed in scale units differing from the mean of the normal. Following saline the mean size at the different times of measurement varied between 0.06 to minus 0.12 scale units. The maximum mydriatic effects (within 315 min.) following different doses of the compounds are shown in fig. 4. It can be seen that all compounds elicit mydriasis. However, the time courses of the mydriatic effects showed different features. Following atropine in a dose of 0.1 to 0.5 mg/kg, maximum mydriasis was obtained at the first measurement after which the response gradually diminished. A similar effect was seen following compound 5 (0.05–0.50 mg/kg). On the other hand, following compounds 3, 8, 9, 10, 11 and 12 there was a slow increase in response during the five-hour experimental periods. In the case of compounds 10, 11 and 12, the pupil diameter

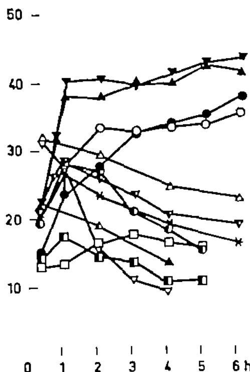


Fig. 5. Mydriatic effect in mice. Intraperitoneal injection of 5 mg/kg. Ordinate: Scale units.

Symbols for compounds:

▽ = 1	◐ = 5	* = 9
△ = 2	▲ = 6	○ = 10
□ = 3	▼ = 7	▼ = 11
◧ = 4	● = 8	▲ = 12

was measured 24 hours after the injection when a further increase was obtained. The other compounds elicited their maximum response within 45 to 135 min. and the effects remained rather constant during 5 hours. Effects

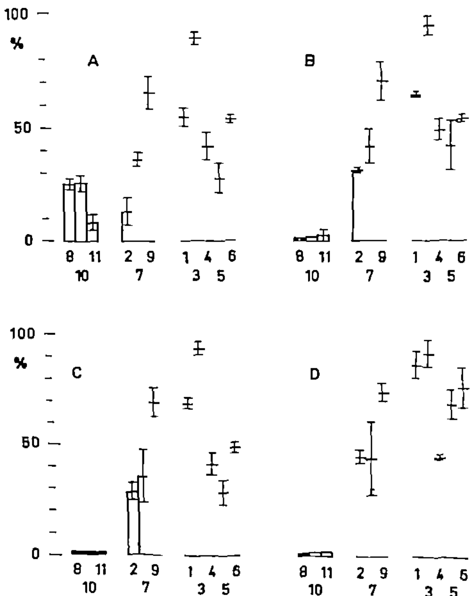


Fig. 6. Blood pressure response of AcCh following compound 1 to 11 (29.5 nmol/kg intravenously). Normal response of AcCh = 100 %. Doses of AcCh (µg/kg intravenously) used at the different times after compounds: A. 10 min. 0.75 B. 20 min. 0.50 C. 30 min. 0.25 D. 60 min. 0.25. Vertical bars: extreme values in 2 to 3 experiments. Numbers refer to compounds in fig. 1.

on the light reflex of the eye are also shown in table 3. Following compounds 1 to 6, 11 and 13 there was a total block of the reflex. A normal or sometimes a slower response was the only effect obtained after compounds 7, 8, 9, 10 and 12 with threshold doses for behaviour impairment. Since there were some difficulties in obtaining exact measurements of the pupil diameter in the dogs due to their unwillingness to be restrained during the behavioural effects of the drugs, the mydriatic effect was also studied in mice. Fig. 5 shows the effects following injection of the same dose (5 mg/kg) of the compounds. In this species too compounds 3–8, 10, 11 and 12 have a slow mydriatic effect which increases over 3 or 6 hours. Atropine, on the other hand, has a maximum response after 20 min. When comparing the response 4 to 6 hours after the injection it is evident that the compounds of group one have the most potent mydriatic effects while those forming group two have somewhat stronger effects than of group three. Following local application, similar effects were obtained.

In order to elucidate the relative cholinolytic potency of the compounds, their effects on the blood pressure response to acetylcholine (AcCh) were studied in the cat, following an equimolar dose (29.5 nmol base/kg intravenously). The results are illustrated in fig. 6 A–D where the dose and schedule of AcCh are given. It is seen that all compounds reduce the response to AcCh but their potencies and time-courses are different. The most

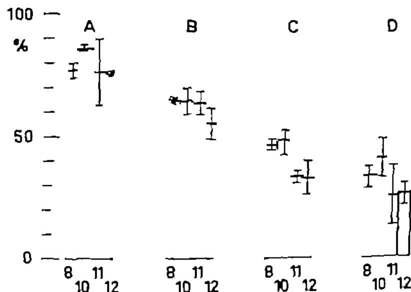


Fig. 7. Blood pressure responses of AcCh following compounds 8, 10, 11 and 12 (5.9 nmol/kg intravenously). Schedule and doses of AcCh correspond to those given in fig. 6.

marked inhibitions were obtained following compounds 8, 10 and 11 (group one on behaviour). Ten min. after the injection only 30 % of the normal AcCh response remained and later on there was a total block. The next potent compounds are 2 and 7 (group two) and 4 5 and 6 (group 3) Much weaker effects are produced by atropine (1) and compounds 3 and 9 The duration is best seen when comparing the effect 30 and 60 min. after injection when the same dose of AcCh was used. At 30 min. after injection (C) compounds 2, 4 5 6 and 7 have about the same inhibitory effects (50–70 %) Thirty min. later (D) the inhibitory effect of compounds 5 and 6 was 30 % but following 2, 4 and 7 it was 55 %

Since the most potent compounds produced a complete inhibition of the AcCh response they were also studied at a 5 times lower dose (5 9 nmol/kg intravenously). The results which are shown in fig. 7 (12 is added), reveal that no major difference in potency and duration could be demonstrated between these substances. In contrast to the less active drugs in fig. 6, the inhibition following compound 8, 10 11 and 12 showed an increasing trend between 30 and 60 min.

Due to the different time-courses of the effects it is difficult to obtain a ratio for the relative potency of the compounds. However if the effects are compared 60 min. after the injection, another series of experiments revealed that the doses of atropine and scopolamine have to be 18 and 6 times higher respectively then that of the most active compounds, in order to obtain the same effect.

Discussion

The results in the present paper have shown that, following the administration of several aryl- and cycloalkyl-aryl glycolates, the same behavioural disturbances are elicited in dogs as those following atropine and scopolamine. The pattern of symptoms corresponds to those reported earlier following atropine-like substances (White *et al.* 1961 Gershon & Bell 1963 Bell & Gershon 1964 Albanus *et al.* 1969) The symptoms are highly reproduceable and this is partly explained by the homogenous dog material. Contrary to reports in the literature pertaining to atropine and scopolamine, no increased tolerance could be observed.

An interesting difference in the effects between the compounds is the time course for the mydriatic effect. The extremely slow effect of some compounds is not seen for the other parameters studied. The same time-course is also obtained following local application on one eye. This indicates that the effect is not a central one. This view is further supported by the observation that the opposite eye remained normal in size and thus no evidence of systemic

REFERENCES

Abood, L. G. The psychotomimetic glycolate esters. In. *Drugs effecting the central nervous system.* Vol. 2. Ed. Burger A. Marcel Dekker Inc., New York, 1968.

Abood, L. G. & J. H. Biel. Anticholinergic psychotomimetic agents. *Int. Rev. Neurobiol.* 1962, 4 217–273

Albanus, L., A. Sundwall, B. Vangbo & B. Winbladh: The fate of atropine in the dog. *Act. pharmacol. et toxicol.* 1968 26 571 582.

Albanus, L., S. M. Aquilonius, A. Sundwall & B. Winbladh. The fate of intracerebroventricular injection of atropine and methylatropine in relation to their pharmacological effects. *Acta pharmacol. et toxicol.* 1969 27 81–96.

Bell, C. & S. Gershon: Experimental anticholinergic psychotomimetics. Antagonism of yohimbine and tacrine (THA). *Med. exp.* 1964 10 15–21

Bell, C., S. Gershon, B. Carrol & G. Holan. Behavioural antagonism to a new psychotomimetic: JB-329 *Arch. Int. Pharmacodyn.* 1964 147 9–25

Franke, S. Psychochemische Kampfstoffe (Psychogifte). In: *Lehrbuch der Militärchemie* Band 1 Deutscher Militärverlag, Berlin, 1967 450.

Gershon, S. Behavioural effects of anticholinergic psychotomimetics and their antagonists in man and animals. *Recent advances biol. psychiat.* 1966, 8, 141 149

Gershon, S. & C. Bell. A study of the antagonism of some indole alkaloids to the behavioural effects of Ditran. *Med. exp.* 1963 8 15–27

Herz, A., H. Teschemacher A. Hofstetter & H. Kurz: The importance of lipid-solubility for the central action of cholinolytic drugs. *Int. J. Neuropharmacol.* 1965, 4 207–218.

Nyberg, K., B. Östman & G. Wallerberg: Investigations of dithienylglycolic esters I *Acta Chem. Scand.* 1970 in press.

Pulewka, P. Das Auge der weissen Maus als pharmakologisches Testobjekt. I Mitteilung: Eine Methode zur quantitativen Bestimmung kleinster Mengen Atropin und anderer Mydriatika. *Arch. exp. Path. Pharmakol.* 1932, 168, 307–318.

White, R. P. C. B. Nash, E. J. Westerbeke & J. Possanza. Phylogenetic comparison of central actions produced by different doses of atropine and hyoscine. *Arch. int. Pharmacodyn.* 1961 132, 349–363

on the light reflex of the eye are also shown in table 3 Following compounds 1 to 6, 11 and 13 there was a total block of the reflex. A normal or sometimes a slower response was the only effect obtained after compounds 7 8 9 10 and 12 with threshold doses for behaviour impairment. Since there were some difficulties in obtaining exact measurements of the pupil diameter in the dogs due to their unwillingness to be restrained during the behavioural effects of the drugs, the mydriatic effect was also studied in mice. Fig. 5 shows the effects following injection of the same dose (5 mg/kg) of the compounds. In this species too, compounds 3 8 10 11 and 12 have a slow mydriatic effect which increases over 3 or 6 hours. Atropine, on the other hand has a maximum response after 20 min. When comparing the response 4 to 6 hours after the injection it is evident that the compounds of group one have the most potent mydriatic effects while those forming group two have somewhat stronger effects than of group three. Following local application, similar effects were obtained.

In order to elucidate the relative cholinolytic potency of the compounds, their effects on the blood pressure response to acetylcholine (AcCh) were studied in the cat, following an equimolar dose (29.5 nmol base/kg intravenously). The results are illustrated in fig. 6 A–D where the dose and schedule of AcCh are given. It is seen that all compounds reduce the response to AcCh but their potencies and time-courses are different. The most

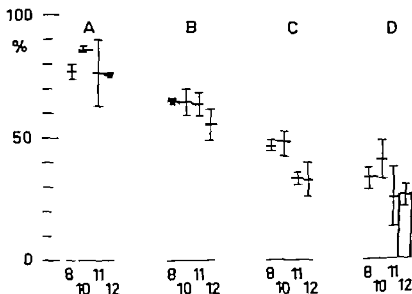


Fig. 7 Blood pressure response of AcCh following compounds 8, 10, 11 and 12 (3.9 nmol/kg intravenously). Schedule and doses of AcCh correspond to those given in fig. 6.

marked inhibitions were obtained following compounds 8, 10 and 11 (group one on behaviour). Ten min. after the injection only 30 % of the normal AcCh response remained and later on there was a total block. The next potent compounds are 2 and 7 (group two) and 4 5 and 6 (group 3) Much weaker effects are produced by atropine (1) and compounds 3 and 9 The duration is best seen when comparing the effect 30 and 60 min. after injection when the same dose of AcCh was used. At 30 min. after injection (C) compounds 2, 4 5 6 and 7 have about the same inhibitory effects (50–70 %) Thirty min. later (D) the inhibitory effect of compounds 5 and 6 was 30 % but following 2, 4 and 7 it was 55 %

Since the most potent compounds produced a complete inhibition of the AcCh response they were also studied at a 5 times lower dose (5 9 nmol/kg intravenously) The results which are shown in fig 7 (12 is added) reveal that no major difference in potency and duration could be demonstrated between these substances. In contrast to the less active drugs in fig. 6, the inhibition following compound 8, 10 11 and 12 showed an increasing trend between 30 and 60 min.

Due to the different time-courses of the effects it is difficult to obtain a ratio for the relative potency of the compounds. However if the effects are compared 60 min. after the injection, another series of experiments revealed that the doses of atropine and scopolamine have to be 18 and 6 times higher respectively then that of the most active compounds, in order to obtain the same effect.

Discussion

The results in the present paper have shown that, following the administration of several aryl- and cycloalkyl-aryl glycolates, the same behavioural disturbances are elicited in dogs as those following atropine and scopolamine. The pattern of symptoms corresponds to those reported earlier following atropine-like substances (WHITE *et al* 1961 GERSHON & BELL 1963 BELL & GERSHON 1964 ALBANUS *et al.* 1969) The symptoms are highly reproduceable and this is partly explained by the homogenous dog material Contrary to reports in the literature pertaining to atropine and scopolamine, no increased tolerance could be observed.

An interesting difference in the effects between the compounds is the time course for the mydriatic effect. The extremely slow effect of some compounds is not seen for the other parameters studied. The same time-course is also obtained following local application on one eye. This indicates that the effect is not a central one. This view is further supported by the observation that the opposite eye remained normal in size and thus no evidence of systemic

absorption was found. The explanation might be that these compounds react slowly with the receptor of this target organ.

The most potent drugs with regard to effects on behaviour (group one) i.e. diphenyl thienyl-phenyl and dithienyl substituted quinuclidinyl glycolate (compounds 8 10 11 and 12), elicited their effect following doses of 10 µg/kg. They are also the most potent compounds with regard to the anticholinergic effects as revealed by their effect on the heart rate and the inhibition of AcCh response on blood pressure. The threshold dose for effects on the heart rate and behaviour is the same moreover there appears to be a direct correlation between the heart rate and the degree of behavioural impairment. Moreover in the case of scopolamine, piperidyl benzilate and quinuclidinyl phenyl-cyclopentyl glycolate (compound 2, 7 and 9), a relationship between the effects on behaviour and heart rate, is obvious. These compounds are about 5 times less potent and their effects much more short lasting than those in group one.

Atropine, ditran® tropyl benzilate and tropyl and quinuclidinyl-hydroxyfluorene carboxylate (compounds 1 3 4 5 and 6), on the other hand, produce anticholinergic effects in doses far below those which produce effects on behaviour Atropine causes a marked tachycardia (60 % increase) without causing any behavioural effects, and at the threshold dose for ataxia atropine and compound 4 cause a 2–3 times higher increase in heart rate than the substances in groups one and two Methyl atropine elicits behavioural effects when the dose is about 100 times higher than that producing a significant effect on the heart rate. The fact that methyl atropine can produce effects on behaviour which are indistinguishable from those of atropine is interesting since methyl atropine is often used as a scientific tool to distinguish between central and peripheral anticholinergic effects.

The general impression of the relationship between effects on behaviour (especially locomotion) and traditional anticholinergic effects (especially an increase in heart rate) is that they are closely correlated except for a few compounds which are known to be rather lipid-insoluble.

If the potency in blocking the AcCh response on blood pressure is taken as a measure of the anticholinergic potency it is evident that the compounds of group one form a very uniform group, about 6 times more potent than scopolamine and 18 times more potent than atropine However the threshold doses for effect on locomotion are 10 50 and 500 µg/kg respectively Hence the activity of atropine does not seem to be directly correlated to the behavioural effect. This would appear logical since the drug has been shown to penetrate rather slowly into the brain (ALBANUS *et al.* 1968)

The importance of lipid solubility for the relationship between peripheral and central effects of anticholinergic drugs has recently been stressed by HERZ *et al* (1965). The partition coefficients between oleyl alcohol and a

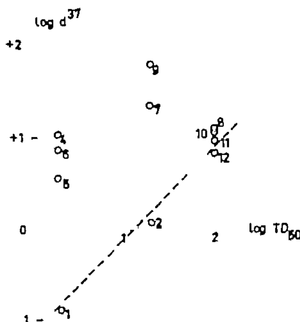


Fig. 8. Logarithm of the partition coefficient (log d^{37} oleyl alcohol/water pH 7.4 37°) plotted against the negative logarithm of behavioural effects in dogs (− log TD_{50}). Dotted line indicates a correlation for some of the compounds. Numbers refer to compounds in fig. 1

phosphate buffer of pH 7.4 have therefore been determined for the compounds, studied in this paper (ALBANUS *et al.* unpublished). Fig. 8 shows the relationship between partition coefficient and threshold dose for effects on behaviour. As seen in the figure above, a correlation is evident for atropine, scopolamine and the compounds in group one. This is surprising since these compounds differ so much in anticholinergic potency. It is obvious that there is no simple relationship between either potency or lipid solubility and effects on behaviour.

An especially shortlasting effect on behaviour is seen following scopolamine (2) and compounds 7 and 9 at the threshold dose. Among these drugs only scopolamine, which has a strong cholinolytic activity, fits the curve in fig. 8.

Nevertheless, the interpretation of the results must be that the effect on behaviour are related to the anticholinergic effects of the drug, but that the lipid solubility and cholinolytic potency are not the only factors which determine the effect on the central nervous system. Obviously several factors interfere with the central activity and one factor of importance is the rate of metabolism.

REFERENCES

Abood, L. G. The psychotomimetic glycolate esters. In *Drugs affecting the central nervous system*. Vol. 2. Ed. Burger A. Marcel Dekker Inc., New York, 1968.

Abood L. G. & J. H. Biel. Anticholinergic psychotomimetic agents. *Int Rev Neurobiol* 1962, 4 217–273.

Albanus, L., A. Sundwall, B. Vangbo & B. Winbladh. The fate of atropine in the dog. *Acta pharmacol. et toxicol.* 1968, 26, 571 582.

Albanus, L., S.-M Aquilonius, A. Sundwall & B Winbladh The fate of intracerebroventricular injection of atropine and methylatropine in relation to their pharmacological effects. *Acta pharmacol. et toxicol.* 1969 27 81–96.

Bell, C. & S. Gershon. Experimental anticholinergic psychotomimetics: Antagonism of yohimbine and tacrine (THA). *Med. exp* 1964, 10 15–21.

Bell, C., S. Gershon, B. Carrol & G Holan: Behavioural antagonism to new psychotomimetic: JB-329 *Arch. int Pharmacodyn.* 1964 147 9–25

Franke, S. Psychochemische Kampfstoffe (Psychogifte). In: *Lehrbuch der Militär chemie* Band 1 Deutscher Militärverlag, Berlin, 1967 450.

Gershon, S. Behavioural effects of anticholinergic psychotomimetics and their antagonists in man and animals. *Recent advances biol. psychiat.* 1966, 8, 141–149

Gershon, S. & C. Bell A study of the antagonism of some indole alkaloids to the behavioural effects of Ditran *Med exp* 1963 8, 15–27

Herz, A., H. Teschemacher A. Hofstetter & H. Kurz: The importance of lipid-solubility for the central action of cholinolytic drugs. *Int J Neuropharmacol.* 1965, 4 207 218.

Nyberg, K., B. Östman & G Wallerberg: Investigations of dithienylglycolic esters I *Acta Chem. Scand.* 1970, in press.

Pulewka, P Das Auge der weissen Maus als pharmakologisches Testobjekt. I. Mitteilung: Eine Methode zur quantitativen Bestimmung kleinster Mengen Atropin und anderer Mydriatika. *Arch. exp Path. Pharmak* l. 1932, 168, 307–318.

White, R. P C. B. Nash, E. J Westerbeke & J Possanza. Phylogenetic comparison of central actions produced by different doses of atropine and hyoscine. *Arch. int Pharmacodyn.* 1961 132, 349–363.

Acta pharmacol. et toxicol. 1970, 28, 327–333

From the Department of Pharmacology
and the Pharmacological Division of the Department of Pharmacy
University of Helsinki, Helsinki, Finland

Inhibition of Energy Production by Halothane Metabolites

By

Per H. Rosenberg, Mauno M. Airaksinen and Tapani Tammisto

(Received November 20, 1970)

Abstract. Trifluoroethanol (TFE), trifluoroacetaldehyde hydrate (TFAIH) and trifluoroacetic acid, which are metabolites of halothane and fluroxene, were given intraperitoneally to mice. TFE, in lethal doses of 300 and 600 mg/kg caused significant decrease of the ATP content and the ATP/ADP ratio in mice livers 17 hrs after the administration. TFE, however did not decrease the content of creatine phosphate (nor the creatine phosphate/creatine ratio) in mice hearts or muscles. All the three halothane metabolites inhibited anaerobic glycolysis (14–72 %) in the liver, the greatest inhibition occuring 17 hrs after the administration of TFAIH. *In vitro* only TFE and TFAIH were found to be inhibitors. Ethanol, which protects against the toxicity of TFE, did not modify the inhibition of glycolysis *in vivo* but totally prevented the decrease in the ATP/ADP ratio. The inhibition of glycolysis as well as the discrepancy between the changes in ATP and creatine phosphate concentrations may be explained by an inhibition by trifluoroacetaldehyde or its derivative of enzymes with active thiol groups.

Key-words. Halothane – alcohol, ethyl – adenine nucleotides.

Trifluoroethanol (TFE) has been reported to be a metabolite of halothane (Van Dyke & Chenoweth 1965) and of fluoroxene (Blake *et al.* 1967). Of the halothane absorbed 17–20 % is metabolized in the human body (Rehder *et al.* 1967). The percentage metabolized through TFE is unsettled (Airaksinen 1968). The major urinary metabolite of halothane is trifluoroacetic acid (TFAA), which may also be formed through another suggested pathway involving an unstable ionized compound (Stier 1967). Thus Cohen & Hood (1969) as well as the present authors (Airaksinen *et al.* unpublished) have found that unidentified metabolites accumulate in the liver after halothane anaesthesia.

We have reported that the LD50 of TFE in mice is not many times higher than the amount of TFE that might be formed after halothane

anaesthesia (AIRAKSINEN & TAMMISTO 1968) Independently BLAKE *et al.* (1969) have reported similar findings, with only a slightly higher LD50 value.

The toxicity of the substance seemed to be caused by a metabolite of TFE (probably trifluoroacetaldehyde or its derivative) which is bound to thiol groups (AIRAKSINEN *et al.* 1970) This might lead to an extensive metabolic disturbance which involves the energy production. Indeed, it was found that the lactate-pyruvate pool in mouse liver and the ATP content in guinea pig muscle was decreased after TFE administration (AIRAKSINEN & TAMMISTO 1968) It thus seemed desirable to study the energy production after the administration of TFE and other known metabolites of halothane.

Material and methods

Male Swiss albino mice weighing 17-20 g were used in all experiments.

Drugs: Trifluoroethanol (TFE) (Fluka AG Buchs SG), trifluoroacetaldehyde hydrate (TFAH) (Pierce Chem. Co. Rockford, Illinois), trifluoroacetic acid sodium (TFAA) (Schuchard, München), trichloroethanol (TCE) (Fluka AG Buchs SG) and ethanol (Alko OY Helsinki).

Determination of adenosine phosphates.

The livers were removed from non-anaesthetized mice as quickly as possible and dropped into liquid nitrogen. The livers were then weighed frozen and pulverized at -20 or below with 10 % trichloroacetic acid, and homogenized in a glass homogenizer after thawing in the refrigerator The ATP ADP and AMP concentrations were measured enzymatically by the method of ADAM (1965) using the reagent kits of Boehringer (Mannheim)

Determination of creatine phosphat and creatine.

The mice were anaesthetized with ether and the tissue samples put as quickly as possible by means of cooled forceps, into liquid nitrogen and then treated like those taken for the adenosine phosphate determinations.

Creatine phosphate was measured spectrophotofluorometrically (CONN 1960) after hydrolysis to free creatine from mouse heart and thigh muscle. Quantitative hydrolysis of creatine phosphate was obtained with 0.1 N-HCl (9 min. at 65°).

Determination of anaerobic glycolysis.

The anaerobic glycolysis was measured by the liberation of CO_2 from Krebs-Ringer bicarbonate solution (UMBREIT *et al.* 1957) containing 0.2 % of glucose. Small flasks of 5.5 ml volume were used, the atmosphere consisted of gaseous mixture of N_2 and CO_2 (95 and 5 %) and the temperature was 37 The flasks were saturated with the gaseous mixture for 15 min. before the experiments.

The mice were decapitated and weighed amounts of the liver were cut with scissors into very small pieces and placed into the flasks. After thermo-equilibrium was obtained within 15 min. the manometers were read at 15 min. intervals for 90 min. The liberation of CO_2 in µl/100 mg of tissue/hr was used for the calculation of the effect of the drugs. For each drug 4 manometers were used and 2 manometers for the standard activity were used in each experiment.

Table 1

Effect of ethanol treatment on the changes in ATP ADP and AMP (μmol/g ± S.E.M.) caused by TFE in mouse liver The animals were sacrificed 17 hrs after intraperitoneal administration of TFE. Ethanol was given intraperitoneally 10 min. before and 3 hrs after TFE. Five experiments for each value.

	ATP	ADP	AMP	ATP/ADP
Controls	1.09 ± 0.14	0.071 ± 0.006	0.060 ± 0.008	13.90 ± 3.75
TFE 300 mg/kg	0.86 ± 0.01	0.109 ± 0.005	0.050 ± 0.003	6.42 ± 0.31
TFE 300 mg/kg + ethanol 500 mg/kg × 2	0.87 ± 0.04	0.078 ± 0.008	0.062 ± 0.004	11.22 ± 0.98**
TFE 600 mg/kg	0.73 ± 0.12	0.120 ± 0.006	0.051 ± 0.006	4 45 ± 1.15
TFE 600 mg/kg + ethanol 500 mg/kg × 2	1 07 ± 0.12	0.088 ± 0.008	0.074 ± 0.010	11.30 ± 1.04
Ethanol 500 mg/kg × 2	1.02 ± 0 09	0 070 ± 0.012	0 063 ± 0.004	14.11 ± 1.20

differs significantly from controls ($P < 0.05$)
differs significantly from TFE 300 mg/kg ($P < 0.01$)
** differs significantly from TFE 600 mg/kg ($P < 0\ 001$)

Table 2

Effect of TFE (300 mg/kg intraperitoneally) on the concentrations of creatine phosphate and creatine (μmol/g ± S.E.M.) of the thigh muscle and heart of mice 5 hrs and 17 hrs after administration. Six mice for each value.

	Creatine phosphate	Creatine	Creatine phosphate/ creatine
MUSCLE			
Controls	6.59 ± 0.70	12.45 ± 1 21	0.56 ± 0.02
TFE 5 hrs	7.06 ± 0.88	13.15 ± 1.01	0.57 ± 0.11
TFE 17 hrs	5.84 ± 0.60	11 13 ± 0.88	0.53 ± 0.04
Atropine + neostigmine* controls	*5.56 ± 0.28*	*11 42 ± 0.60*	*0.54 ± 0.03*
TFE + atropine + neostigmine 5 hrs	6.04 ± 0.13	12.15 ± 0.49	0.50 ± 0.02
TFE + atropine + neostigmine 17 hrs	4 97 ± 0.56	8.93 ± 0.63	0.56 ± 0 09
HEART			
Controls	6.84 ± 1.00	8.94 ± 0 68	0.80 ± 0.14
TFE 5 hrs	7 18 ± 0 86	8.14 ± 0.53	0.89 ± 0.12
TFE 17 hrs	7.09 ± 0.63	10.88 ± 0 56	0.65 ± 0.06

To induce muscl fasciculations atropine (0.5 mg/kg intraperitoneally) and neostigmine (0.1 mg/kg intraperitoneally) were given two hours before killing.

Results

Effect on adenosine phosphates

TFE (300 and 600 mg/kg intraperitoneally) caused a significant decrease in the ATP/ADP ratio in mice livers (table 1). There was both a decrease of the ATP content and an increase of the ADP content, both of which were statistically significant following a dose of 600 mg/kg of TFE 17 hrs before sacrifice. The AMP level was unchanged.

Ethanol prevented both the decrease in ATP and the increase in ADP

Effect on creatine phosphate and creatine.

There were no significant changes in the content of the creatine phosphate (nor in the creatine phosphate creatine ratio) in the heart or thigh muscle after TFE administration to normal or atropine + neostigmine treated mice (table 2) In the latter animals however TFE, seemed to decrease the total content of creatine.

Effect on anaerobic glycolysis.

Lethal and non-lethal doses of TFE and TFAIH, as well as comparable doses of TFAA all had an inhibitory effect on glycolysis *in vivo* the greatest (72.3 %) being with TFAIH administered 17 hrs before sacrifice (fig. 1) The inhibitory effect of TFE occurred within 5 hrs and continued longer than that of the other drugs, remaining almost unchanged for several days or until death of the animal. *In vivo* high doses of ethanol showed a weak inhibition of glycolysis (fig. 1) while TCE was almost as effective as TFE, though shorter in action.

In vitro TFE and TFAIH significantly inhibited the glycolysis (table 3), but TFAA and TCE were without effect. Ethanol did not modify the inhibition produced by TFE.

Discussion

Though a significant decrease in tissue ATP content occurs simultaneously with the toxic symptoms, no definite causal relationship could be ascertained. In guinea pig muscle a decrease in ATP was already found 4 hrs after the administration of TFE, but the same dose of the non-toxic TFAA was ineffective (AIRAKSINEN & TAMMISTO 1968)

The decrease in the concentration of ATP (and the ATP/ADP ratio) after TFE administration seems to be due to the inhibition of glycolysis rather than to disturbances in oxidative phosphorylation, since no inhibition of succinate respiration or uncoupling of oxidative phosphorylation could be observed in rat liver mitochondria *in vitro* with any of the halothane metabolites (ROSEN-

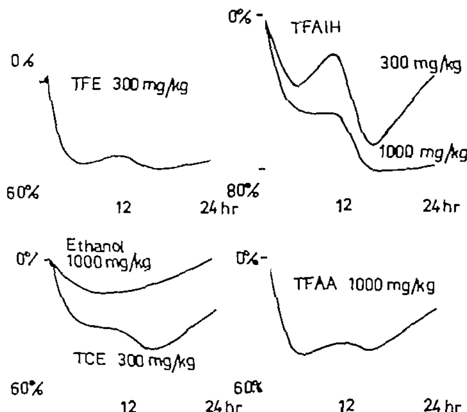


Fig. 1. Effect of *in vivo* administration of TFE, TFAIH, TFAA and ethanol on anaerobic glycolysis in mouse liver homogenates. The per cent inhibition at 5 12, 17 and 24 hrs was measured. At least four livers for each value.

Table 3

The per cent inhibition (± S.E.M.) of anaerobic glycolysis by TFE, TFAIH, TFAA, TCE and ethanol in mice liver homogenates *in vitro*. At least 4 livers for each value.

	10^{-6} M	10^{-4} M	$10^{-[illegible]}$ M	$10^{-[illegible]}$ M
TFE	(35.43 ± 6.21)	29.36 ± 6.35	37.14 ± 5.12	32.15 ± 10.35
TFAIH	(0)	12.18 ± 4.01	24.81 ± 4.23	19.64 ± 1.83
TFAA	0	0	0	0
TCE	0	0	0	0
Ethanol	33.40 ± 15.90	23.83 ± 7.33	20.16 ± 6.46	–
TFE + ethanol**	34.72 ± 6.91	20.96 ± 6.24	28.74 ± 4.23	–

In high concentrations evaporation of the drugs may have caused the determinations to be invalid.

** TFE and ethanol at equal concentrations.

BERG & WIKSTRÖM unpublished). The inhibition of glycolysis may not, however, be the only cause of the decrease in ATP as ethanol, which effectively protects against the toxicity of TFE (AIRAKSINEN *et al.* 1970; BLAKE *et al.* 1969) prevented the decrease in ATP but did not prevent the inhibition of glycolysis by TFE.

Extreme metabolic changes occurred *in agone* affect glycolysis, but its inhibition also occurred before the toxic symptoms. The *in vivo* inhibition by the non toxic agent TFAA seemed to be non-specific and of short duration, like that of alcohols (ethanol and TCE).

Our toxicological studies have indicated that trifluoroacetaldehyde formed *in vivo* might well contribute to the toxicity of TFE (AIRAKSINEN *et al.* 1970). TFE did not, in fact, inhibit glycolysis in the brain and heart tissue (AIRAKSINEN *et al.* 1969) or affect the growth of fibroblasts *in vitro* (ROSENBERG & WAHLSTRÖM 1970), all being tissues with low alcohol dehydrogenase activity (FAZEKAS & RENGEI 1968) in which aldehyde cannot be formed.

Enzymes with active thiol groups, e.g. creatine kinase (ATP-creatine phosphate transferase), are partly inactivated when these groups react with aldehyde groups (MADSEN 1963). The SH inhibiting property of trifluoroacetaldehyde thus might explain the discrepancy between the decrease in the ATP/ADP ratio and the unchanged creatine phosphate/creatine ratio, since the change in energy rich phosphates is usually first recognized in the creatine phosphate content (GUTTON 1966). The inhibition of glycolysis is probably explained by a similar mechanism.

Acknowledgements

The study was supported in part by grants from the Foundation for Alcohol Studies in Finland and the National Research Council for Natural Sciences, Finland.

The authors wish to thank Mrs. Ritva Aarnuharju and Mrs. Katarina Mäkynen for technical assistance.

REFERENCES

Adam, H.: Adenosine-5-triphosphate determination with phosphoglycerate kinase, pp. 539–543. In: *Methods in enzymatic analysis*. Ed. by H. U. Bergmeyer. Verlag Chemie, Weinheim and Academic Press, New York 1965.

Airaksinen, M. M.: Toxicity of the metabolites of halothane. IV World Congress of Anaesthesiologists. London 1968. *Exc. Med. Int. Congr. Ser.* No. 200, pp. 326–328.

Airaksinen, M. M., P. H. Rosenberg & T. Tammisto: Ethanol and the toxic actions of halothane metabolites. Proceedings of IV International Congress on Pharmacology. Basel 1969.

Airaksinen, M. M., P. H. Rosenberg & T. Tammisto: A possible mechanism of the toxicity of trifluoroethanol and other halothane metabolites. *Acta pharmacol. et toxicol.* 1970, 28, 299–304.

Airaksinen, M. M. & T. Tammisto: Toxic actions of the metabolites of halothane. LD50 and some metabolic effects of trifluoroethanol and trifluoroacetic acid in mice and guinea pigs. *Ann. Med. exp. Fenn.* 1968, 46, 242–248.

Blake, D. A., H. F. Cascorbi, R. S. Rozman & F. J. Meyer: Animal toxicity of 2,2,2-trifluoroethanol. *Toxicol. Appl. Pharmacol.* 1969, 15, 83–91.

Blake, D. A., R. S. Rozman, H. F. Cascorbi & J. C. Krantz Jr.: Anaesthesia. LXXIV Biotransformation of fluroxene-I. Metabolism in mice and dogs in vivo. *Biochem. Pharmacol.* 1967, 16, 1237–1248.

Cohen, E. N. & Nancy Hood: Application of low-temperature autoradiography to studies of the uptake and metabolism of volatile anesthetics in the mouse. III Halothane. *Anesthesiology* 1969, 31, 553–559.

Conn, R. B. Jr.: Fluorometric determination of creatine. *Clin. Chem.* 1960, 6, 537–548.

Pareiras, L. Gy. & B. Rengel: Über die Alkoholdehydrogenase-Aktivität der Organe normaler und alkoholbehandelter Ratten. *Enzymologia* 1968, 34, 231–236.

Guyton, A. C.: *Textbook of medical physiology.* W. B. Saunders Co., Philadelphia and London. 1966.

Madsen, N. B.: Mercaptide-forming agents, pp. 119–143. In: *Metabolic inhibitors.* Ed. by R. M. Hochster & J. H. Quastel. Vol. II, Acad. Press, New York and London 1963.

Rehder, K., J. Forbes & H. Alter: Halothane biotransformation in man. A quantitative study. *Anesthesiology* 1967, 28, 711–715.

Rosenberg, P. H. & T. Wahlstrom: Hepatotoxicity of halothane metabolites *in vivo* and inhibition of fibroblast growth *in vitro*. *Acta pharmacol. et toxicol.* 1970, 28, in press.

Stier, A.: The biotransformation of halothane. *Anesthesiology* 1967, 29, 388–389.

Umbreit, W. W., R. H. Burris & J. F. Stauffer: Manometric techniques. 3. ed. Burgess Publ. Co. Minneapolis. 1957.

Van Dyke, R. A. & M. B. Chenoweth: Metabolism of volatile anesthetics. *Anesthesiology* 1965, 26, 348–357.

Acta pharmacol. et toxicol 1970 28 334–337

From the Section of Biochemistry (A. Lund, Ph. D.), Institute
of Forensic Medicine, University of Copenhagen
(Professor H. Gormsen, M. D.)

Concentrations of Meprobamate in the Blood and Liver Following Fatal Meprobamate Poisoning

By

S. Felby

(Received February 27 1970)

Abstract The concentrations of meprobamate in the blood and liver were determined in 12 cases of death due to meprobamate poisoning and in 29 cases of death due to the combined effects of meprobamate and alcohol, meprobamate and barbiturate, or meprobamate, alcohol and barbiturate. In the cases of poisoning from meprobamate alone, the blood meprobamate concentrations were within the range of 142–342 µg/ml (mean value: 226 µg/ml). In the cases of poisoning with a combination of the drugs, the blood meprobamate concentrations were: for meprobamate and alcohol, 43–155 µg/ml (mean value 117 µg/ml); for meprobamate and barbiturate, 30-276 µg/ml (mean value 117 µg/ml)· for meprobamate, barbiturate and alcohol, 53–460 µg/ml (mean value 133 µg/ml).

Key-words: Meprobamate – poisoning.

Meprobamate (2 methyl 2-propyl-1.3-propandioldicarbamate) has been widely used since 1955 as a tranquillizer when its effect in the treatment of anxiety was first demonstrated. In spite of the enormous consumption of meprobamate, very *few* studies have been reported of determinations of meprobamate concentrations in the blood and tissues following fatal meprobamate poisoning (POWELL *et al.* 1958 FINKLE 1967 MADDOCK & BLOOMER 1967)

We thought it of interest, therefore to make a survey of all the cases of fatal poisoning seen at the Copenhagen Institute of Forensic Medicine during the period 1964–69 during which meprobamate had been either the sole cause of death or together with other sedatives or hypnotics, a major contributory factor

The survey reported here, covers 41 cases, 20 men (aged 19–81) and 21 women (aged 18–70) in which death was due to meprobamate poisoning alone, or to the combined effects of meprobamate and alcohol or barbiturate, or both.

Analytical methods

3.00 ml of blood (or liver homogenized with equal parts of water) is shaken with 25.0 ml of chloroform, and the chloroform extract then shaken with 5.00 ml of 0.5 N ammonia. The extraction with chloroform is repeated with a further 3.00 ml of blood, and the chloroform shaken with 0.5 N sodium hydroxide. The alkaline extracts are then used for spectrophotometric determination of barbiturat (maxima at 240 mμ (NH_3) or 255 mμ (NaOH)). The chloroform extract is dried with anhydrous sodium sulphate and used for determination of meprobamate. 1.0–5.0 ml of the dried chloroform extract is evaporated on a water bath. A red colour is developed on the residue, with 4-dimethylaminobenzaldehyde and antimony trichloride (HOFFMAN & LUDWIG 1959).

Alcohol concentrations in the blood were determined enzymatically using the ADH method.

Results and discussion

It can be seen from table 1 that in 12 cases death was due to poisoning from meprobamate alone. The blood meprobamate concentrations were within the range of 142–346 μg/ml (mean value 226 μg/ml). By way of comparison it may be mentioned that therapeutic daily doses of 1.2–1 6 g of meprobamate yield concentrations of 10–20 μg/ml. The lethal blood meprobamate concentrations seen in this study were of the same order of magnitude as those reported by other investigators. POWELL *et al.* (1958) in two cases of fatal meprobamate poisoning, found meprobamate in the liver amounting to 220 μg/g and 300 μg/g, respectively FINKLE (1967) in one case, found a blood meprobamate concentration of 194 μg/ml and MADDOCK & BLOOMER (1967) also in one case, found a blood meprobamate concentration of 174 μg/ml. The last-mentioned investigators stated that blood meprobamate concentrations exceeding 100 μg/ml bring about a deep coma.

The cases in the table, are listed in descending order of blood meprobamate concentrations. It can be seen that there is no clear correlation between the concentrations of meprobamate and the concentrations of barbiturate or alcohol, i. e. low meprobamate concentration with high barbiturate or alcohol concentration or vice versa.

In 10 cases of poisoning from the combined effects of meprobamate and alcohol, the blood meprobamate concentrations were within the range of 43–155 μg/ml (mean value 117 μg/ml), the average meprobamate concentration being about one half of that found in poisoning with meprobamate alone. This is in agreement with the observation of other investigators that meprobamate and alcohol are synergistic in their central nervous depressant action (GOLDBERG 1961).

In the 12 cases in which death was due to the combined effects of meprobamate and barbiturate, the blood meprobamate concentrations w

Table 1

Concentrations of meprobamate and barbiturate in the blood (µg/ml) and liver (µg/g), and the concentrations of alcohol in the blood (mg/g) following fatal poisoning in human subjects.

Intake of meprobamate (g)	Meprobamate		Alcohol Blood	Barbiturate		Lapse of time from absence of symptoms until found dead (hours)
	Blood	Liver		Blood	Liver	
	346					
	313					
	294	208				
20	276	412				
	220	204				
	207	255				26
32–40	200	224				8
20	187	147				
	178					14
32	171	245				
17	170	254				22
16–20	142	197				7
Mean	226 µg/ml					
	155		0.71			18
20	147	231	0.71			5
	139	102	1.00			17
	137	158	2.12			10
32	111		1.33			
	111		0.19			16
	110	174	1.29			
	109	208	0.89			18
20	103		1.42			
	43		2.68			
Mean	117 µg/ml					
	276	604		19	54	
	224	312		29	91	10
	160	320		12	13	
	153	137		13	81	
	131	168		33	51	
	115	189		135	179	
	88	102		39	49	7
	80	357		40	193	10
	56	67		20	58	
	53	148		58		9
	42			15		10
	30	109		31	69	
Mean	117 µg/ml					

	460		0.65	57		
	152	140	1.98	22	25	9
	97	65	1.89	69	71	3
	88	151	0.65	96	149	
	62	137	0.44	9	29	
	39	52	0.52	41	78	
	33	32	0.67	26	40	
Mean	133 μg/ml					

within the range of 30–276 μg/ml (mean value 117 μg/ml) and in the seven cases of mixed meprobamate barbiturate and alcohol poisoning, the blood meprobamate concentrations were within the range of 33–460 μg/ml (mean value 133 μg/ml). In spite of the considerable deviation in the blood concentrations of meprobamate seen in this material, the mean values clearly show the synergism between the central nervous depressant actions of meprobamate and barbiturate, as well as between meprobamate, barbiturate and alcohol. In the cases of combined poisoning the average lethal blood meprobamate concentration was half that observed in poisoning with meprobamate alone.

According to the data available, the interval between the time at which the subjects had last been seen alive, without showing any symptoms of poisoning, and the time when they were found dead was 3–26 hours (last column of the table). Poisoning from meprobamate alone, or poisoning from the combined effects of meprobamate and the above mentioned substances may thus result in death within a very short time. There is scanty information available about the amounts of meprobamate taken in subjects with suicidal intent; it appears, however, that the minimum lethal dose in man is about 20 g (first column of the table).

REFERENCES

Finkle, B. S.: The identification, quantitative determination, and distribution of meprobamate and glutethimide in biological material. *J. forensic Sci.* 1967, 12, 509–528.

Goldberg, L.: Alcohol, tranquillizer and hangover. *Quart. J. Stud. Alcohol.* 1961, Suppl. 1, 37–56.

Hoffman, A. J. & B. J. Ludwig: An improved method for the determination of meprobamate in biological fluids. *J. Am. Pharm. Assoc.* 1959, 48, 740–742.

Maddock, R. K. & H. A. Bloomer: Meprobamate overdosage. Evaluation of its severity and methods of treatment. *J. Am. med. Ass.* 1967, 201, 123–127.

Powell, L. W., G. T. Mann & S. Kay: Acute meprobamate poisoning. *New Eng. J. Med.* 1958, 259, 716–718.

Acta pharmacol et toxicol. 1970 28, 338–345

From the Department of Pharmacology[1] and the Department of Pathology[2]
B J Medical College, Poona, India

Effect of Thyroxine, Reserpine and Serotonin on Allyl Alcohol Induced Hepatotoxicity in Rats*

By

S. Srinivasan[1] U. Srinivasan[2] and J H. Balwani[1]

Received January 9 1970

Abstract Rats, treated with allyl alcohol (AA), 0.8 % w/v 4 ml/kg, orally (group B) and examined 24 hrs later showed an increase in the levels of serum *bromsulfphthalein (BSP)* (3 ×) and *glutamic-pyruvic transaminase (GPT)* (5 ×) and periportal hepatic necrosis, as compared with the control (group A). Treatment with thyroxine sodium (T_4), 1.6 mg/kg given orally daily for 6 days (group C) raised the rectal temperature by about 2 Reserpine, 5 mg/kg *intraperitoneally (group D) or serotonin, 5 mg/kg intraperitoneally (group E)* lowered the rectal temperature by 1–2 within 1 hr Groups C, D and E had normal hepatic function and morphology When group B was treated with T_4 as mentioned above (group F), the increase in BSP and GPT levels (both 8 ×) and the degree of necrosis were more marked. When group B was pretreated with reserpine (group G) or serotonin (group H) 1 hr before AA, the increase in serum BSP (about 1.5 × and 2.5 × respectively) and GPT (about 3.5 × and 4 × respectively) and the degree of necrosis were less pronounced. When group F was also treated with reserpine (group I) the rise in body temperature was reduced from about 2 to about 1 while the serum BSP and GPT levels and the degree of necrosis were brought to near group B levels. When group F was also treated with serotonin (group J) however there was no reduction in body temperature, the serum BSP and GPT levels, or the degree of necrosis. These findings point to possible direct relationship between body temperature and the hepatotoxic ffect of AA.

Key-words. Thyroxine – reserpine – serotonin – allyl alcoh l hepatotoxicity

Calvert & Brody (1961) demonstrated a potentiating effect of thyroxine on the hepatotoxic effect of carbon tetrachloride. A similar effect of thyroxine on the hepatotoxic effect of thioacetamide was shown by Srinivasan & Balwani (1968) The latter also showed a correlation between the hyper

---

Work presented t the Second Annual Conference of the Indian Pharmacological Society held at Lucknow 1969

thermic effects of thyroxine and its potentiation of carbon tetrachloride and thioacetamide toxicity and suggested that thyroxine might increase the susceptibility of the liver cell to toxic attack, irrespective of the mechanism of the latter. It was, therefore, decided to study the effect of hyperthermia (produced by thyroxine) and hypothermia (produced by reserpine and serotonin) on the hepatotoxic effect of allyl alcohol.

Materials and Methods

Stock male albino rats, weighing 90–140 g, were used. The body temperature of the animals was recorded by means of rectal thermometer. Allyl alcohol, 0.8 % (w/v), 4.0 ml/kg was given orally and the extent of liver damage was studied 24 hours later by means of bromsulfphthalein excretion, serum levels of glutamic-pyruvic transaminase and histological examination. Bromsulfphthalein excretion was studied by the method previously described by SRINIVASAN & BALWANI (1968). The blood was collected from the neck vessels under light ether anaesthesia. Serum levels of glutamic-pyruvic transaminase were estimated by the method of REITMAN & FRANKEL (1957) as described by WOOTTON (1964). The enzyme levels were expressed in international units (I. u.). A piece of liver tissue was examined histologically for the extent of necrosis after staining with haematoxylin and eosin.

To induce hyperthermia, thyroxine sodium, 1.6 mg/kg, was administered orally to the rats every day for 6 days and allyl alcohol was given 24 hours after the last dose of thyroxine. In order to produce hypothermia, reserpine (5 mg/kg intraperitoneally) or serotonin (5 mg/kg intraperitoneally) was given 1 hour before the administration of allyl alcohol. In other studies, reserpine or serotonin was also given to rats pretreated with thyroxine as mentioned above, 1 hour before the administration of allyl alcohol.

The control animals received saline in appropriate amounts. The liver function was also studied in animals receiving thyroxine, reserpine, or serotonin only in the doses mentioned above.

All the experiments were conducted at room temperature (15–25°) during the rainy season and early winter. Statistical comparisons were made by Student's t-test for comparing means.

Results

Body temperature

Treatment with thyroxine daily for 6 days significantly raised the rectal temperature of the rats. A single intraperitoneal injection of reserpine or serotonin significantly lowered the rectal temperature of the rats within 1 hour and this was maintained at the lower level for the next 24 hours. Reserpine produced a more marked fall in rectal temperature than serotonin.

Reserpine antagonized the hyperthermic effect of pretreatment with thyroxine whereas serotonin failed to do so. Allyl alcohol by itself did not alter the body temperature of the rats, with or without pretreatment (table 1).

Liver function studies

Rats treated with allyl alcohol showed significantly increased levels of glutamic-pyruvic transaminase and retention of bromsulfphthalein in the

Table 1

Body temperature of rats.

Group	Body temperature C ± S.E.M.	
1. Blank control	(10) 37 74 ± 0.42	
2. Allyl alcohol	(10) 37.81 ± 0.58	1 vs 2 NS
3 Thyroxine	(8) 39 78 ± 0.66	1 vs 3 S
4. Reserpine	(8) 35.83 ± 0.85	1 vs 4 S
5 Serotonin	(8) 36.08 ± 1.10	1 vs 5 S
6. Thyroxine + allyl alcohol	(10) 39.81 ± 0.68	2 vs 6 S
7 Reserpine + allyl alcohol	(9) 35.55 ± 0.89	2 vs 7 : S
8. Serotonin + allyl alcohol	(9) 36.10 ± 0.81	2 vs 8 S
9 Thyroxine + reserpine + allyl alcohol	(10) 38.28 ± 0.49	6 vs 9 S
10. Thyroxine + serotonin + allyl alcohol	(7) 39.95 ± 0.57	6 vs 10 NS

Figures in brackets indicate number of observations.
S Significant ($P < 0.05$) NS Not significant.

Table 2

Liver function tests.

Group	Serum glutamic-pyruvic transaminase levels (I. u./litre) ±S.E.M.	Serum bromsulfphthalein levels mg % ± S.E.M.	P
1. Blank control	(10) 31 ± 6	(10) 1.9 ± 0.8	
2. Allyl alcohol	(10) 160 ± 21	(10) 7.2 ± 1.1	1 vs 2 S
3 Thyroxine	(8) 34 ± 10	(8) 2.1 ± 0.8	1 vs 3 NS
4. Reserpine	(8) 29 ± 8	(8) 2.0 ± 0.8	1 vs 4 NS
5. Serotonin	(8) 35 ± 7	(8) 2.1 ± 0 9	1 vs 5 NS
6. Thyroxine + allyl alcohol	(10) 239 ± 23	(10) 15.9 ± 3.3	2 vs 6 S
7 Reserpine + allyl alcohol	(8) 110 ± 18	(9) 3.3 ± 0.9	2 vs 7 : S
8. Serotonin + allyl alcohol	(9) 119 ± 20	(9) 4.8 ± 1.0	2 vs 8 : S
9 Thyroxine + reserpine + allyl alcohol	(9) 145 ± 34	(10) 8.0 ± 1.2	6 vs 9 : S
10. Thyroxine + serotonin + allyl alcohol	(7) 216 ± 27	(7) 13.2 ± 3.0	6 vs 10 NS

Figures in brackets indicate number of observations.
S Significant ($P < 0.001$) NS : Not significant.



Fig. 1. Rat liver 24 hours after administration of allyl alcohol. Haematoxylin and eosin.

serum. Histologically there was necrosis of hepatic cells around the portal tracts but the centrolobular region was unaffected (table 2 & fig. 1).

Rats pretreated with thyroxine showed more pronounced liver damage after allyl alcohol, as evidenced by a further increase in the blood levels of transaminase and bromsulphthalein and a more marked necrosis (fig. 2). On



Fig. 2. Rat pretreated with thyroxine. Liver 24 hours after administration of allyl alcohol. Haematoxylin and eosin.

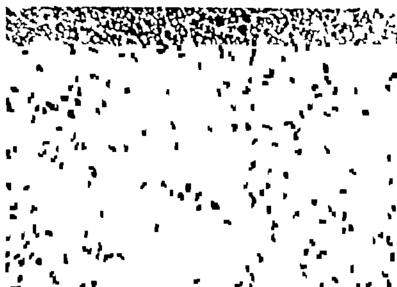


Fig. 3. Rat pretreated with reserpine. Liver 24 hours after administration of allyl alcohol. Haematoxylin and eosin.

the other hand, pretreatment with reserpine or serotonin reduced the liver damage by allyl alcohol, as shown by the significantly lower levels of transaminase and bromsulfphthalein in the serum and also by a less marked necrosis (fig. 3 & 4).



Fig. 4 Rat pretreated with serotonin. Liver 24 hours after administration of allyl alcohol. Haematoxylin and eosin.



Fig. 5. Rat pretreated with thyroxine and reserpine. Liver 24 hours after administration of allyl alcohol. Haematoxylin and eosin.

When rats pretreated with thyroxine for 6 days were also treated with reserpine or serotonin 1 hour before a challenge dose of allyl alcohol, it was found that reserpine significantly antagonized the hepatotoxic-potentiating effect of thyroxine, whereas serotonin had no effect (fig. 5 & 6)



Fig. 6. Rat pretreated with thyroxine and serotonin. Liver 24 hours after administration of allyl alcohol. Haematoxylin and eosin.

Thyroxine, reserpine or serotonin alone did not alter the hepatic function tests or the histology of the liver

Discussion

CALVERT & BRODY (1961) showed a potentiating effect of the hepatotoxic effect of thyroxine on carbon tetrachloride SRINIVASAN & BALWANI (1968) extended the similarity between carbon tetrachloride and thioacetamide by demonstrating a similar potentiation of thioacetamide toxicity by thyroxine. These latter workers also suggested that there was a correlation between the hyperthermic metabolic effect of thyroxine and its potentiation of the hepatotoxic effect. They suggested that the effect of thyroxine might be non-specific, i. e. it might affect other hepatotoxic agents in a similar manner

In the present study thyroxine has been shown to augment the liver damage produced by another hepatotoxin viz. allyl alcohol, which produces a periportal type of necrosis, unlike that of thioacetamide and carbon tetrachloride which damage the cells in the centrolobular areas. This finding may be interpreted as giving further support to a possible general effect of thyroxine on hepatotoxins.

As pretreatment with thyroxine also raised the body temperature by about 2 the effect of a lowered body temperature was studied using reserpine and serotonin. Phenothiazines were avoided as they are known to protect the liver against toxic effects (REES *et al* 1961) It was found that both serotonin and reserpine lowered the body temperature significantly and also reduced the liver damage produced by allyl alcohol.

Moreover it was of interest to study the effect of these hypothermic drugs on thyroxine pretreated animals. Here, however reserpine and serotonin behaved differently While reserpine antagonized both the hyperthermic and hepatotoxic-potentuating effects of thyroxine, serotonin did not have either of these effects. This further points to a direct relationship between the body temperature of the animals and the extent of liver damage produced by the hepatotoxin.

While thyroxine may produce hyperthermia by its characteristic metabolic effects and the hypothermic effect of reserpine may be mediated by a depletion of biogenic amines at critical sites in the central nervous system, the hypothermic effect of systemically administered serotonin is yet to be explained, since it crosses the blood-brain barrier but poorly (FELDBERG 1966) The divergent effects of reserpine and serotonin on thyroxine hyperthermia also remain to be explained. A thyroid-catecholamine relation (HARRISON 1964) may be the basis for the reserpine antagonizing effect of thyroxine hyperthermia. Since the hypothermia of systemically administered serotonin

is itself poorly understood, no speculations can be offered for the failure of serotonin to produce hypothermia in thyroxine treated animals.

Whatever the mechanisms involved, the present findings indicate a direct relationship between body temperature and the hepatotoxic effect of allyl alcohol. Possibly the susceptibility of the metabolic processes in the liver cell to toxic attack may be increased by the accelerated metabolism at higher body temperatures and reduced by the slowed metabolism at lower body temperatures.

Acknowledgements

The authors wish to thank Dr G V Joglekar for his helpful suggestions, Dr R. V Junnarkar for kindly providing facilities for histological work and microphotography and Dr Y M. Bhende, the Dean, B J Medical College and Sassoon General Hospitals for his encouragement during the work.

REFERENCES

Calvert, D N. & T M. Brody: The effects of thyroid function upon carbon tetrachloride hepatotoxicity *J Pharmac. Exp Therap.* 1961, 134 304–310.

Feldberg, W S.. The monoamines of the hypothalamus as mediators of temperature responses. In. *Recent advances in pharmacology* Ed. Robson, J M. and R. S Stacey J & A. Churchill Ltd., London. 1968, pp. 349–397

Harrison, T S.. Adrenal medullary and thyroid relationships. *Physiol. Rev* 1964 44 161 185.

Rees, K. R., K. P Sinha & V G. Spector: The pathogenesis of liver injury in carbon tetrachloride and thioacetamide poisoning. *J Path. Bact.* 1961, 81 107–118.

Reitman, S. & S. Frankel A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. *Amer J clin. Path.* 1957 28 56–63.

Srinivasan, S. & J H. Balwani: Effect of thyroxine on thioacetamide hepatotoxicity *Acta pharmacol. et toxicol.* 1968, 26, 475–481.

Wootton, I. D. P *Microanalysis in medical biochemistry* J & A. Churchill Ltd., London, 1964, p. 112.

Acta pharmacol. et toxicol. 1970 28 346-358

From the Department of Clinical Chemistry (University of Edinburgh)
Royal Infirmary Edinburgh, Scotland

Paraquat Poisoning

By

S. L. Tompsett

(Received March 5 1970)

Abstract. This paper deals with the development of methods for the detection and determination of the weed-killer paraquat, in body fluids (urine, blood, gastric aspirate, dialysates) and tissues. These have been obtained from 6 cases of paraquat poisoning (2 of which were fatal) admitted to hospital and from several other negative cases. A specific colour reaction is described for the detection and determination of paraquat. Thus when sodium hydrogen carbonate followed by sodium dithionite is added to the test solution, a blue colour is produced. The detection limit is about 1 microgram/ml. Following admission to hospital shortly after the ingestion of paraquat, the reaction can be applied directly to diluted urine and diluted (filtered) gastric aspirate. Paraquat can be detected in small quantities in urine for quite a long period after ingestion. It is necessary however to use a column of a cation exchange resin to achieve concentration and also to remove any interfering substances. Under these conditions, it is preferabl to assess the absorbance of the blue colour on recording spectrophotometer The concentration of paraquat in the blood serum is extremely low and only detectable shortly after ingestion. It has been extremely difficult to detect any traces of paraquat in dialysates (haemo- and peritoneal) Depending on the interval of time between ingestion and death, paraquat may be detected in some tissues e. g liver and kidney Using the cation exchange resin technique, paraquat in quantities of 50 to 100 micrograms added to 100 ml of urine can be recovered with an accuracy of 86 to 91 %

Key-words. Poisoning – toxicity

Paraquat (1 1 -dimethyl-4 4 -dipyridyl, 2A) is an extensively used and efficient weed killer In the United Kingdom it can be supplied as a concentrated solution (gramoxone W®) for agricultural purposes or admixed with solid magnesium sulphate (weedol®) for use in domestic gardens. The concentrate may contain at least 20 / paraquat whereas the domestic solid preparation contains only 5 / paraquat. A concentrated solution containing a related compound viz. diquat (reglone®) is also available for the same

purpose in agriculture. Paraquat and diquat can be used in mixture in agriculture.

Cases of paraquat poisoning have resulted from the accidental ingestion of the concentrate – gramoxone W® – from bottles intended for soft drinks. Some cases have been associated with suicidal intent. Ingestion of weedol® has usually been associated with suicidal intent.

Studies on paraquat toxicity in experimental animals have been made by CLARK *et al.* (1966) and DANIEL & GAGE (1966) but these unfortunately bear little relationship to the accidental and suicidal poisonings that have occurred in man.

The toxicity of ingested paraquat has been established but the minimum lethal dose in man has still to be determined (MATTHEW *et al* 1968) The features of paraquat poisoning in man are renal, hepatic and pulmonary toxicity A number of fatal cases have been reported. There is an extensive literature on the clinical and pathological aspects of the subject (BULLIVANT 1966 ALMOG & TAL 1967 MOURIN 1967 OREOPOULOS *et al* 1968 CAMPBELL 1968 KERR *et al.* 1968 MATTHEW *et al.* 1968). The last publication included the use of lung transplantation in an attempt to save the life of young patients.

Chemical data relating to paraquat poisoning do not appear to have been available until the publication of the chemical studies of KERR *et al.* (1968) and MATTHEW *et al.* (1968). The chemical studies involving six definite cases of paraquat poisoning and several suspected but negative cases have now been performed by the present authors. The chemical results obtained from these cases are recorded in Case histories

It has been suggested on the basis of animal experiments (DANIEL & GAGE 1966), that ingested paraquat is rapidly excreted, mainly in the urine, and that any subsequent pathological changes that may occur could be based on the "hit and run aspects of the substance. That ingested paraquat can be eliminated in small quantities for quite a long period has been demonstrated in this laboratory (MATTHEW *et al* 1968 the present paper). The generally accepted method of treatment is to expedite the elimination of paraquat as quickly as possible. This has been achieved by normal urinary excretion, forced diuresis, peritoneal dialysis and haemodialysis. The last is essential if renal failure becomes a predominant symptom.

The admission of suspected cases of paraquat poisoning to hospital, has produced a number of problems, particularly with regard to its toxicity and some of the irreversible pathological changes that may occur (MATTHEW *et al.* 1968). Chemical control in the treatment of such cases would appear to be indispensable.

This paper is concerned with methods for the detection and determination of paraquat in biological materials e. g. urine, gastric contents and tissues.

The development of these techniques has been associated with the study of both known and suspected cases of paraquat poisoning admitted to hospital. Brief details have been given in previous reports (KERR *et al.* 1968 MATTHEW *et al.* 1968 TOMPSETT 1968a, b c & 1969)

Materials and Methods

Chemical procedures have been described for the determination of both paraquat and diquat in food residues and herbicide preparations (CALDERBANK *et al.* 1961; CALDERBANK & YUEN 1965 YUEN *et al.* 1967) They involve the digestion of the food residue with dilute sulphuric acid, followed by filtration. The filtrate is diluted with water and then applied to a column of Zeo-Karb 225 (containing approximately 8 % divinyl benzene), both paraquat and diquat being retained. Paraquat and diquat may then be eluted with a saturated aqueous solution of ammonium chloride. Paraquat and diquat are then determined colorimetrically in the eluate by the addition of an alkaline solution of sodium dithionite. With paraquat, an intense blue colour is produced and with diquat, a yellowish green colour Both are highly sensitive and specific reactions.

Although paraquat and diquat possess sensitive and characteristic UV spectra, UV spectrophotometry is not readily applied for their detection and determination in biological material because of the difficulties in separation from interfering material. Although these two substances may be readily separated by the use of ion exchange resins, they are extremely water soluble and cannot be separated by the use of organic solvents.

The following are modifications of the bove procedure which have been found to be more suitable for use in the examination of material derived from cases of paraquat poisoning admitted to hospital.

Colorimetric determination of paraquat

To 5 ml of fluid is added 0.1 g of sodium hydrogen carbonate followed by 0.1 g of sodium dithionite. A blue colour develops almost immediately

For quantitative measurement, absorbance are read at 625 mm. The following is a suitable range of standards – 2.5 to 20 micrograms of paraquat/mL The intensity of the colour remains unchanged for at least 1 hour

The intensity of the blue colour obtained with given quantity of paraquat is the same irrespective of whether sodium hydrogen carbonate or sodium carbonate is used or whether the original alkaline dithionite reaction is used.

The UV spectra and the spectra of the coloured products produced in the presence of dithionite are shown in fig. 1 to 4.

Separation of paraquat from biol gical fluids and extracts by means of an ion exchange resin.

This procedure is an extension of the one studied in relation to the separation of many other substances of pharmacological and toxicological interest (TOMPSETT 1960, 1961, 1962, 1963, 1968a, b & c TOMPSETT *et al* 1961).

The ion exchange material was in the form of a column and had the following characteristics

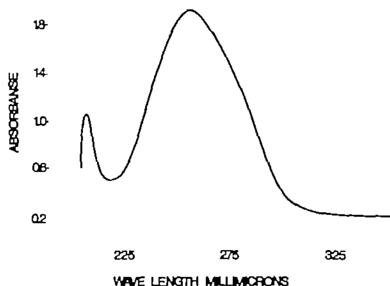


Fig. 1. U V spectrum. Paraquat. 25 μg/ml N hydrochloric acid.



Fig. 2. U V spectrum. Diquat. 25 μg/ml N hydrochloric acid.

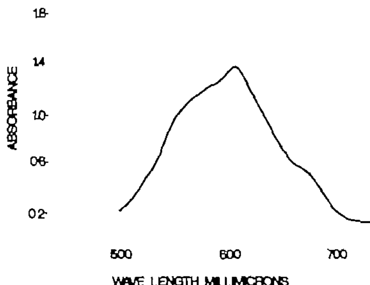


Fig. 3. Paraquat. Dithionite reaction. 40 μg/ml.

Material. Dowex 50 W × 12 (mesh 200/400).
Weight. 3 g.
Chromatography assembly manufactured by Quickfit and Quartz Ltd., England, with 10 × 100 mm column and sintered glass base.
Dimensions of effective column
Height: 70 mm.
Diameter: 70 mm.

Before use, the column is washed with 100 ml of 8 N hydrochloric acid and then with water until the eluate is neutral. Several of these columns are kept in readiness for use.

100 ml of the biological fluid, to which has been added 10 ml of 10 N hydrochloric acid, are applied to the column. The column is then washed with 100 ml of N hydrochloric acid followed by 100 ml of 2.5 N hydrochloric acid. The retained paraquat is then eluted with 100 ml of 5 N hydrochloric acid. The eluate is evaporated to dryness in an all glass vacuum distillation apparatus. The residue is dissolved in a small volume of water. Finally the colorimetric reaction with sodium hydrogen carbonate and sodium dithionite is applied to an aliquot of this solution.

The examination of various biological materials.
Gastric aspirate.

Such material is usually obtained shortly after ingestion and relatively high concentrations are encountered. Under such conditions, the gastric aspirate may be filtered or filtered after dilution with water and the colorimetric reaction applied to an aliquot of the filtrate. Various dilutions may be required in order to bring the intensity of the blue colour within the range of the standards. Interference from the presence of background colour is rarely encountered.

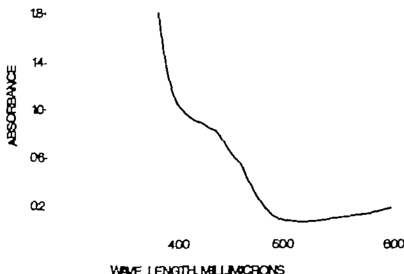


Fig. 4 Diquat. Dithionite reaction. 200 μg/ml.

Urine.

A considerable range in concentration may be found and this can determine the type of technique used.

1. Admission to hospital shortly after the ingestion of paraquat, results in the excretion of urine with a relatively high concentration of this substance. Under such conditions, the direct reaction may be applied and usually considerabl dilution with water is necessary in order that the intensity of the colour produced is within the range of the standards. Under these conditions there is no interference from background colour

2. As the concentration of paraquat in urine decreases with time after ingestion, the presence of paraquat by the application of the direct colorimetric reaction is still detectable though the presence of background colour makes quantitative assessment difficult.

3. At a later period, the presence of traces of paraquat is undetectable by the use of the direct colorimetric reaction.

Under the conditions prevailing in (2) and (3), more satisfactory results can be achieved by the use of an ion exchange resin which not only results in increased concentration but also removes most of the background colour material.

The concentration of paraquat in urine below which it is advisable t use the ion exchange concentration technique depends on many factors e.g. the initial colour of the urine. In general, the critical level is approximately 200 micrograms/100 ml.

Cation exchange technique

10 ml of 10 N hydrochloric acid are added to 100 ml of urine and the mixture applied to column as described above. The technique is then continued to completion as described above.

When concentrations are low some interference may occur from the presence of background colour This can be eliminated by the use of recording spectrophotometer

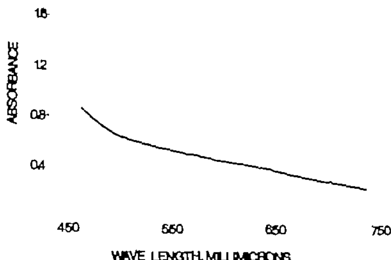


Fig. 5 Concentrated urine extract from cation exchange resin column. Dithionite reaction. Paraquat absent.

(Unicam SP 800). Background coloration usually has a linear slope and under these conditions, correction is relatively simple (see fig. 5 & 6).

Paraquat, in quantities of 50 to 200 micrograms, added to 100 ml of urine, could be recovered with an accuracy of 86–91 % when the ion exchange technique was used.

Ion exchange resin procedures can be slow. In cases of emergency it has been found possible to speed up the flow of fluid through the column by the use of a water suction pump, without detracting from the accuracy of the results. Under such conditions, a result can be obtained within 3 hours e. g. 50 micrograms paraquat/100 ml urine.

Blood

Blood levels are low of the order of no more than 100 micrograms/100 ml even when urinary levels can be expressed in mg/100 ml. The determination is more of interest than useful. The following procedure has been used.

To 10 ml of whole blood or blood serum are added 20 ml of 20 % trichloroacetic acid and the mixture diluted to 100 ml by the addition of water. The mixture is centrifuged. The volume of the centrifugate is noted and then applied to an ion exchange column. The procedure is then completed as described above.

20 and 50 micrograms of paraquat added to 10 ml whole blood could be recovered with an accuracy of 71–82 %

Tissues

Since these are obtained from a post-mortem examination, the concentrations encountered will depend on the interval between ingestion and death. Up to the present these have been found to be low or non-detectable. The following procedure has been used.

20 ml of homogenised tissue, 20 ml of water and 10 ml of concentrated hydrochloric acid are placed in stoppered flask. Th flask is placed in a boiling water bath for 1

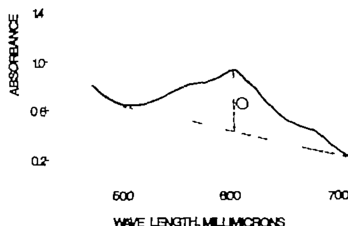


Fig. 6. Concentrated urine extract from cation exchange resin column. Dithionite reaction. Paraquat present. A = Absorbance due to paraquat.

hour and then cooled. The contents of the flask are diluted to 100 ml by the addition of water. The mixture is filtered. The volume of the filtrate is noted and it is then applied to an ion exchange resin column. The procedure is then completed as described above.

Haemo- and peritoneal dialysates.

The concentrations of paraquat in these fluids, if detectable, are very low. The procedure described below was used.

A mixture of 100 ml of fluid and 10 ml of 10 N hydrochloric acid is applied to an ion exchange column. The procedure is completed as described above.

Diquat.

The author has not, as yet, had any experience in the examination of material derived from patients admitted to hospital after the ingestion of diquat. The method of approach however would be similar to that used for paraquat though the colour evaluation could be more difficult (c.f. fig. 3 & 4). Using the ion exchange resin technique, diquat is recovered in the same manner and in the same fraction as paraquat.

Case histories

Case 1

A man (aged 32 years) was admitted to hospital after having ingested about 45 g of weedol® (containing 5 % paraquat) for suicidal purposes. The treatment included forced diuresis. Outcome – not fatal.

Gastric aspirate (on admission)
Paraquat – 21.5 mg/100 mL
Urine (on admission)
Volume – 75 mL
Paraquat – 14.8 mg/100 mL

Time (hours)	*Urine*		*Blood Serum*
	Volume (ml)	*Paraquat* (µg/100 ml)	*Paraquat* (µg/100 ml)
0– 2	1775	840	85 (0 hours)
2– 4	575	510	40 (4 hours)
4– 6	750	490	
6– 8	950	330	
8–10	900	230	40 (10 hours)
10–12	950	230	
12–14	500	230	
14–16	1100	105	
16–18	700	95	not detectable
18–20	850	95	not detectabl
20–22	1400	95	not detectable
22–24	650	95	not detectable

Case 2

A youth (aged 15 years) accidentally ingested gramoxone W® from a bottle labelled "Kola The amount swallowed at the most was one mouthful. The youth was treated locally and was later removed to a city hospital. Forced diuresis was undertaken locally and again on admission to the city hospital. A lung transplantation was carried out but the youth died, 18 days after ingestion. For further clinical details, see MATTHEW *et al.* (1968).

The following are the chemical data.

Urine.

(1) Urine collected up to 14 hours after ingestion – 99 mg paraquat/total.
(2) Urine collected during the first period of forced diuresis – 102 mg paraquat/total.
(3) Urine collected during the second period of forced diuresis – 3 mg paraquat/total. (blood paraquat – 25 micrograms/100 ml).
(3) Urine collected daily from the second to eighteenth day after ingestion.

Days after ingestion	*mg paraquat/total*
2	4.1
3	2.2
4	0.8

5	3.4
6	4.4
7	12.3
9	3.8
11	4.0
12	3.7
13	2.2
14	1.6
15	1.2
16	1.2
17 and 18	not detectable

NOTE there was a copious flow of urine during this period.

Tissues.

(a) Lung removed at transplantation contained 850 micrograms/100 g wet tissue.

(b) Lung(s), kidney(s), liver spleen, bone and brain recovered at P.M. (19 days after ingestion).

Paraquat could not be detected in any of these tissues.

Case 3

A boy (aged 11 years) accidentally ingested a mouthful of gramoxone W® after having taken a heavy meal. The boy was induced to vomit shortly after ingestion. Three specimens of urine became available for examination. Outcome – not-fatal.

Days after ingestion	*Paraquat (μg/100 ml urine)*
7	85
9	30
14	not detectable

Case 4

A young woman (aged 17 years) accidentally took a mouthful of gramoxone W® from a bottle intended for soft drinks. Realising her mistake, she spat it out. She was not admitted to hospital for observation and treatment until 4 days after ingestion. Outcome – not-fatal.

Day after ingestion	*Paraquat (μg/100 ml urine)*
4	50*
5	75
6	140
7	55
8	5
9	15
10	10
11	110
12	125
13	15
14–18	not detectabl

blood paraquat – not detectable.

Case 5

A woman (aged 46 years) accidentally ingested a mouthful of gramoxone W® from a bottle intended for soft drinks. Realising her mistake, she spat it out. She was not admitted to hospital for observation and treatment until 3 days after the episode. Outcome – fatal.

Only small quantities of urine were available and finally tissues obtained at post mortem examination.

Urine no	*Volume (ml)*	*Paraquat (µg/100 ml urine)*
1	8	125
2	5	85
3	7	35

blood paraquat – not detectable.

Tissues (P.M.)	*Paraquat (µg/100 g fresh tissue)*
Liver	20
Lung	not detectable
Kidney	95
Heart	not detectable
Fat	not detectable
Skeletal Muscle	40

Case 6

A man (aged 44 years) was admitted to hospital after having ingested weedol® (containing 5 % paraquat) for suicidal purposes. Outcome – not-fatal.

(Gastric aspirate (on admission)

Paraquat – 2.5 mg/100 ml.

Urine

First day of admission
(various specimens)*

Volume (ml)	*Paraquat (mg/100 ml)*
320	3.8
150	2.5
1250	0.95
1100	0.52

Days after ingestion	*Volume (ml)*	*Paraquat (µg/100 ml)*
2	45	220
3	50	110
14 and 15		not detectable

blood paraquat – 55 µg/100 ml.

Discussion

As far as the clinician is concerned, cases of paraquat poisoning admitted to hospital present a very serious problem. Chemical analyses are essential in order (1) to obtain evidence that paraquat has actually been ingested and (2) to help in the control of treatment.

When patients are admitted to hospital shortly after the ingestion of paraquat, identification represents no difficulty. The test described in this paper has distinct advantages over the alkaline dithionite reaction originally described, since the preparation of an unstable reagent is not required. In fact, the test can be carried out in the hospital ward side room. The most suitable material for the use of this test is urine and/or gastric aspirate.

Patients admitted to hospital after several days following the ingestion of paraquat, represent a different problem. Their symptoms and history could suggest paraquat ingestion but some chemical evidence is required. Gastric fluid will not be available and the urine concentration will be so low that it will be necessary to use a cation exchange resin in order to detect the presence of paraquat.

Although paraquat may appear to be eliminated rapidly the results presented in this paper indicate that paraquat continues to be excreted in the urine in small quantities for quite a long period of time after ingestion.

Blood levels are extremely low in comparison with those found in urine. Blood levels are of interest but are of little value in the diagnosis of the condition.

It has been shown that following death from paraquat poisoning, traces of the substance can be detected in some tissues.

Cases of paraquat poisoning due to the ingestion of either gramoxone W® or solutions of weedol® have occurred in Northern Ireland. Some cases have been accidental and others have been suicidal in intent. Fatalities have occurred. Analyses of materials from these cases have been carried out and the figures obtained were of a similar order to those reported in this paper (CARSON 1969 and personal communication).

A method using thin layer chromatography has been described for the identification of paraquat and diquat in biological material (HEYNDRICKX 1969).

REFERENCES

Almog, Ch., & E. Tal. Death from paraquat after subcutaneous injection. *Brit. Med. J* 1967 3 721.

Bullivant, C. M. Accidental poisoning by paraquat – report of two cases in man. *Brit. Med. J* 1966, 1 1272 1273.

Calderbank, A., C. B. Morgan & S. H. Yuen. Determination of diquat residues in potatoe tubers. *Analyst* 1961 86 569–579
Calderbank, A. & S. H. Yuen. An ion exchange method for determining paraquat in food residues. *Analyst* 1965 90 99–106.
Campbell, S.. Death from paraquat in a child. *Lancet* 1968, 1 144
Clark, D G T F McElligott & E. W Hurst. The toxicity of paraquat. *Brit J Industr Med.* 1966, 23 126–132.
Carson, E. D.: Paraquat poisoning. *Bull. Internat. Assoc. Forens. Toxicol.* 1969 6 No. 2, page 2.
Daniel, J W & J C. Gage: Absorption and excretion of diquat paraquat in rats. *Brit. J Industr Med.* 1966, 23 133–136.
Heyndrickx: Toxicology of insecticides, rodenticides, herbicides and phytopharmaceutical compounds. In. *Progress in Chemical Toxicology* Volume 4 edited by A. Stolman, Academic Press, New York and London, 1969
Kerr F A. R. Patel, P D. R. Scott & S. L. Tompsett: Paraquat poisoning treated by forced diuresis. *Brit. Med. J* 1968, 3, 290–291
Matthew H., A. Logan, M. F A. Woodruff & B Heard. Paraquat poisoning – lung transplantation. *Brit Med. J* 1968, 3, 759–763
Mourin, K. A.. Paraquat poisoning. *Brit Med. J* 1967 4 486.
Oreopoulos, D. G M. A. O. Soyannivo, R. Sinniah, S. S. A. Fenton, M. G. McGeown & J H. Bruce: Acute renal failure in a case of paraquat poisoning. *Brit Med. J* 1968, 1 749–750.
Tompsett, S. L.. The separation of alkaloids from extracts of animal tissues; the use of cation exchange resins. *Acta pharmacol. et toxicol.* 1960, 17 291–303.
Tompsett, S. L.. The separation of alkaloids from extracts of animal tissues; the use of cation exchange resins. *Acta pharmacol. et toxicol.* 1961, 18 414–418.
Tompsett, S. L.. The detection and determination of hypotensive drugs in human serum. *Acta pharmacol. et toxicol.* 1962, 19 365–367
Tompsett, S. L.. The use of a cation exchange resin in the examination of biological materials for substances of physiological, pathological and toxicological interest. 3rd International meeting in forensic medicine, immunology pathology and toxicology April 16–24, 1963, School of Pharmacy London.
Tompsett, S. L.. Detection and determination of paraquat and diquat in urine and gastric contents. *Proc. Assoc. Clin. Biochem.* 1968a, 5 75–76
Tompsett, S. L.. Detection and determination of paraquat and diquat in urine and gastric contents. *Bull. Internat Assoc. Forens. Toxicol.* 1968b, 5 No. 2, page 1.
Tompsett, S. L.. Extraction procedures in chemical toxicology *Analyst* 1968c, 93, 740–748.
Tompsett, S L. Paraquat poisoning. *Bull. Internat Assoc. Forens Toxicol.* 1969, 6, No. 2, page 5
Tompsett, S. L., W Forshall & D C. Smith. The identification and determination of some hypotensive drugs in urine. *Acta pharmacol. et toxicol.* 1961, 18, 75–79
Yuen, S. H., J E. Burgess & D Myles: Spectrophotometric determination of diquat and paraquat in aqueous herbicide preparations. *Analyst* 1967 97 375–381.

Acta pharmacol et toxicol. 1970 28, 359–369

From the Department of Pathology University of Lund, and Sanct Lars Hospital, Lund, Sweden

Subacute and Chronic Trichloroethylene Poisoning
A Neuropathological Study in Rabbits

By

V J Bartonicek and A. Brun

(Received January 12, 1970)

Abstract: Rabbits were given trichloroethylene parenterally in subacute trial lasting for 29 days and in a chronic trial lasting from 41 to 247 days. Behaviour body weight and sedimentation rate were followed. The rabbits were killed at different times during the trial and the brains examined histologically and histochemically for any pathological changes. Moderate changes in the form of diffuse chronic-ischaemic or toxic nerve cell damage in the majority of the cranial nerve nuclei, in the Gasserian ganglion, the cerebellar cortex were demonstrated as well as increased neuronal acid phosphatase activity However no severe neurological disturbances and no changes in the sedimentation rate were noted. The clinically known neurological and/or psychic derangements in humans are discussed in relation to the present pathological findings in rabbits.

Key-words: Trichloroethylene – poisoning.

A detailed systematic examination of the toxic effects of trichloroethylene on the central nervous system has not yet been described. Surprisingly few histopathological studies of animals given chronic doses have appeared during the last two decades. The most extensive report on trichloroethylene is that by ADAMS *et al* (1951) which included examination of the majority of parenchymal organs in different species and over various periods of exposure to trichloroethylene (TRI) with randomly performed examination of the brain and optic nerves. Another study was published by PENNAROLA *et al.* (1966) which included examination of the brain and spinal cord.

Severe and prolonged exposure to TRI has been found in man to result in neurological and/or psychiatric derangements. Thus, neurological syndromes localized to the brain stem structures and/or cranial nerves, such as olfactory optic, oculomotor trigeminal, facial and vestibular nerves, and to the cerebral and cerebellar cortex have been observed. Psychic disturbances as well as organic dementia due to TRI have also been described.

This prompted us to supplement our previous studies on the absorption (BARTONÍČEK 1962), metabolism (BARTONÍČEK & SOUČEK 1959 BARTONÍČEK 1960) and excretion of TRI (BARTONÍČEK & TEISINGER 1962 BARTONÍČEK 1963) with further investigations on the possible pathologic-anatomical effects of TRI on the central nervous system.

Material and Methods

16 female rabbits were divided into three groups. The first group consisting of nine animals (1500–2020 g, average 1770 g) was used for chronic tests; the second group consisting of three animals (1700–1910 g, average 1805 g) was used for subacute tests; the third group consisting of four animals (1700–2700 g, average 2160 g) served as controls and received olive oil only. A fixed dose of TRI per animal per week was administered. Thus, rabbits in the chronic experiment were given 2 ml TRI (= 2.92 g) twice a week, and rabbits in the subacute experiment were given 3 ml (= 4.38 g) three times a week. The duration of the experiment, total amount of the injected TRI, body weight and SR (sedimentation rate) are given in table 1.

TRI was given parenterally by deep intramuscular injection alternately into the right and left gluteal region. It was mixed with equal amount of sterile olive oil. Control rabbits were given sterile olive oil injected in the same manner. In order to check that no inflammatory process was involved the SR was recorded before starting the experiment, monthly and immediately before killing the animals. The reduction in the body weight was used as a rough estimation of the physical condition of the rabbits.

The rabbits were killed or died spontaneously at different times during the trial. In the chronic tests the experiment lasted from 41 to 247 days, i. e. the animals received from 18 g to 133 g of injected TRI. The subacute experiment, on the other hand, had a fixed duration of 29 days and the rabbits each received 53 g of injected TRI. Thus, the weekly loading with TRI was about six times higher in the subacute than in the chronic trial. This experiment enabled us to compare the severity of the toxic effects in the chronically as well as in the subacutely intoxicated groups, and also, in the individual rabbits in the chronically injected group.

TRI used in the experiment (trade mark Mallinckrodt, USA) had the following specification: Max. content Pb 0.00001 % Ni 0.00001 % Cu 0.00001 % Boiling range (95 %) 86.6–87.4 Sp.gr at 25/25 1.459–1.463. Controlled to contain not more than 1 part per million of heavy metals.

The rabbits were killed by decapitation under light intraperitoneal mebumal anaesthesia. The brains, including olfactory bulbs, hypophysis, Gasserian ganglia and eye bulbs with adjacent optic nerves were dissected out and fixed immediately in 4 % formaline for one week at room temperature.

Brain slices of two test animals and one control were in addition used for the demonstration of acid phosphatase on cryostat sections of either unfixed tissue with naphthol-AS-TR phosphate (Sigma) as substrate and pararosaniline (Sigma) as couple (BARKA & ANDERSON 1963, page 245) or of fixed tissue by a modification of the Gomori lead method (BARKA & ANDERSON 1963, page 240).

The tissue samples taken from the eye and optic nerve, from the olfactory nerves and bulbs, the frontal, central and occipital part of the brain, cerebellum, pons and medulla oblongata, trigeminal nerves as well as Gasserian ganglion were embedded in paraffin and sectioned in 6 sections. The stainings used were cresyl-violet, haematoxylin-eosin, according to MAHON (1937) for myelin, and silver impregnation for axons and neurofibrils.

Table 1

Summary of the experimental procedures including several physiological parameters. Rabbits No. S 0, K 02 and K 03 are controls, the others are experimental animals

Rabbit No.	Body wt. at start.	Body wt. at term.	Days of trial	Total TRI g.	Total oil g.	SR start. SR term.	Notes
Subacute exposure to trichloroethylene							
S 0	1700	1870	29	0	33	1/3 2/3	
S 1	1700	1470	28	53	33	2/5 5/7	
S	1820	1660	29	53	33	1/2 4/6	†/
S 3	1910	1640	29	53	33	3/6 5/7	
Chronic exposure to trichloroethylene							
K 01	2350	2780	84	0	23	3/5 4/6	
K 02	1890	3740	262	0	85	2/5 3/6	
K 03	2700	3320	132	0	50	2/3 2/4	
K 4	1500	2650	131	54	34	1/3 2/4	†/
K 5	1650	2600	121	50	32	2/3 12/18	
K 6	1820	2770	180	88	55	0/2 3/5	†/
K 7	1930	2150	41	18	11	0/2 3/4	
K 8	1710	1860	50	22	14	3/8 4/10	†/
K 9	1850	2350	75	32	20	0/1 3/6	
K 10	2020	2180	47	20	13	1/2 2/3	†/
K 11	1580	3480	247	110	69	0/2 3/5	‡/
K 12	1890	3000	247	133	84	1/2 2/6	

Notes: SR start. = sedimentation rate at the start of the trial, 1 and 2 hrs value.
SR term. = sedimentation rate at the end of the trial, 1 and 2 hrs value.
†/ = the rabbit died immediately after injection due to oil embolism.
‡/ = the exposure was interrupted for 42 days.

This prompted us to supplement our previous studies on the absorption (BARTONÍČEK 1962), metabolism (BARTONÍČEK & SOUČEK 1959, BARTONÍČEK 1960) and excretion of TRI (BARTONÍČEK & TEISINGER 1962, BARTONÍČEK 1963) with further investigations on the possible pathologic-anatomical effects of TRI on the central nervous system.

Material and Methods

16 female rabbits were divided into three groups. The first group consisting of nine animals (1500–2020 g, average 1770 g) was used for chronic tests; the second group consisting of three animals (1700–1910 g, average 1805 g) was used for subacute tests; the third group consisting of four animals (1700–2700 g, average 2160 g) served as controls and received olive oil only. A fixed dose of TRI per animal per week was administered. Thus, rabbits in the chronic experiment were given 2 ml TRI (= 2.92 g) twice a week, and rabbits in the subacute experiment were given 3 ml (= 4.38 g) three times a week. The duration of the experiment, total amount of the injected TRI, body weight and SR (sedimentation rate) are given in table 1.

TRI was given parenterally by deep intramuscular injection alternately into the right and left gluteal region. It was mixed with equal amount of sterile olive oil. Control rabbits were given sterile olive oil injected in the same manner. In order to check that no inflammatory process was involved the SR was recorded before starting the experiment, monthly and immediately before killing the animals. The reduction in the body weight was used as a rough estimation of the physical condition of the rabbits.

The rabbits were killed or died spontaneously at different times during the trial. In the chronic tests the experiment lasted from 41 to 247 days, i.e. the animals received from 18 g to 133 g of injected TRI. The subacute experiment, on the other hand, had a fixed duration of 29 days and the rabbits each received 53 g of injected TRI. Thus, the weekly loading with TRI was about six times higher in the subacute than in the chronic trial. This experiment enabled us to compare the severity of the toxic effects in the chronically as well as in the subacutely intoxicated groups, and also, in the individual rabbits in the chronically injected group.

TRI used in the experiment (trade mark Mallinckrodt, USA) had the following specification. Max. content Pb 0.00001 %, Ni 0.00001 %, Cu 0.00001 %. Boiling range (95 %) 86.6–87.4. Sp.gr. at 25/25 1.459–1.463. Controlled to contain not more than 1 part per million of heavy metals.

The rabbits were killed by decapitation under light intraperitoneal mebumal anaesthesia. The brains, including olfactory bulbs, hypophysis, Gasserian ganglia and eye bulbs with adjacent optic nerves were dissected out and fixed immediately in 4 % formaline for one week at room temperature.

Brain slices of two test animals and one control were in addition used for the demonstration of acid phosphatase on cryostat sections of either unfixed tissue with naphthol-AS-TR phosphate (Sigma) as substrate and pararosaniline (Sigma) as coupler (BARKA & ANDERSON 1963, page 245) or of fixed tissue by a modification of the Gomori lead method (BARKA & ANDERSON 1963, page 240).

The tissue samples taken from the eye and optic nerve, from the olfactory nerves and bulbs, the frontal, central and occipital part of the brain, cerebellum, pons and medulla oblongata, trigeminal nerves as well as Gasserian ganglion were embedded in paraffin and sectioned in 6 sections. The stainings used were cresyl-violet, haematoxylin-eosin, according to MAHON (1937) for myelin, and silver impregnation for axons and neurofibrils.

Table 1

Summary of the experimental procedures including several physiological parameters. **Rabbits No. S 0, K 02 and K 03 are controls, the others are experimental animals**

Rabbit No.	Body wt. at start.	Body wt. at term.	Days of trial	Total TRI g.	Total oil g.	SR start. SR term.	Notes
Subacute exposure to trichloroethylene							
S 0	1700	1870	29	0	33	1/3 2/3	
S 1	1700	1470	28	53	33	2/5 5/7	
S 2	1820	1660	29	53	33	1/2 4/6	†/
S 3	*1910*	*1640*	29	53	33	3/6 5/7	
Chronic exposure to trichloroethylene							
K 01	2350	2780	84	0	23	3/5 4/6	
K 02	1890	3740	262	0	85	2/5 3/6	
K 03	2700	3320	132	0	50	2/3 2/4	
K 4	1500	2650	131	54	34	1/3 _/4	†/
K 5	1650	2600	121	50	32	2/3 12/18	
K 6	1820	2770	180	88	55	0/2 3/5	†/
K 7	1930	2150	41	18	11	0/2 3/4	
K 8	1710	1860	50	22	14	3/8 4/10	†/
K 9	1850	2350	75	32	20	0/1 3/6	
K 10	2020	2180	47	20	13	1/2 2/3	†/
K 11	1580	3480	247	110	69	0/2 3/5	‡/
K 12	1690	3000	247	133	84	1/2 2/6	

Notes: SR start. = sedimentation rat at the start of the trial, 1 and 2 hrs value.
SR term. = sedimentation rate at the end of the trial, 1 and 2 hrs value.
†/ = the rabbit died immediately after injection due to oil embolism.
‡/ = the exposure was interrupted for 42 days.

Results

Subacute intoxication

Nerve cells in the telencephalic cortex, basal ganglia and brain stem nuclei in all the animals showed an eosinophilic homogenization of the cytoplasm with slight shrinkage of the cell body and hyperchromasia of the nucleus. These changes were particularly marked in the outer and inner cortical layers, the intermediate layers being relatively unaffected. Around some of these cells an increased number of satellite cells were centered, resembling the picture of neuronophagia. There was no diffuse gliosis.

A number of cerebellar Purkinje cells also revealed these changes. Some of them had disappeared together with the basket cells. Similar alterations were seen in some neurons of the Gasserian ganglion, together with proliferation of the capsular cells.

All experimental animals in this group had multiple infiltrates of lymphocyte like cells in the pons, cerebellum and telencephalon (fig. 1). These cells sometimes formed perivascular collars but were also clustered in the parenchyma. These changes were usually not associated with demyelination.

The control group also showed shrinkage of the cytoplasm and nuclear hyperchromasia, though only in a limited number of cells. Similar changes were observed in the Purkinje cells but there was no drop out of these cells or the basket cells. Such changes are commonly also encountered in untreated controls and are presumably due to terminal events or histotechnical procedures.



Fig. 1. Rabbit medulla oblongata, subacute intoxication: mainly perivascular round cell infiltrations. Haematoxylin-eosin. Magnification × 160.

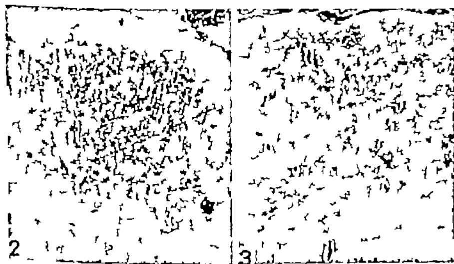
In both groups the olfactory optic and trigeminal nerves and the eye were normal.

The naphthol-AS-RT-phosphate method for acid phosphatases revealed a cytoplasmic granular reaction product in the nerve cells of both groups though in the experimental animals the neurons of the outer and inner cortical layers had a more marked activity than the controls. This difference was more marked with shorter periods of incubation (60 minutes) than with longer periods (150 minutes) With the Gomori method there appeared to be more granules in the neurons of the experimental animals than in the control animals, though the activity was too weak to allow of a definite interpretation.

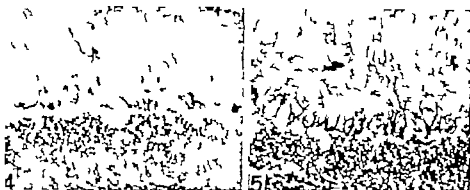
Chronic Intoxication

As in the preceeding group of animals there was a widespread neuronal change consisting of eosinophilic homogenization and shrinkage of the cytoplasm and nuclear hyperchromasia. It was however clearly more pronounced in experimental animals than in the controls and in the subacutely intoxicated rabbits (figs. 2 and 3).

Purkinje cells showed the same type of damage and had in places entirely disappeared together with their basket cells. They were replaced by glial cells. These changes tended to increase in severity with increasing periods of exposure (figs. 4 and 5)



Figs and 3. Rabbit parasagittal frontal neocortex, chronic intoxication shrunken hyperchromatic cortical nerve cells clearly more numerous in test animal (fig. 2) compared to control (fig. 3). Haematoxylin-eosin. Magnification × 70.



Figs. 4 and 5 Rabbit cerebellar cortex, chronic intoxication: loss and degeneration of Purkinje cells and basket cells in test animal (fig. 4) but not in the control (fig. 5). Silver impregnation. Magnification × 160.

In all cases some neurons of the Gasserian ganglion showed similar alterations and proliferation of the capsular cells. In one animal only could the lymphocyte-like infiltrations described in the subacute experiment be seen. The eye, optic, trigeminal and olfactory nerves were normal.

Control rabbits had occasional nerve cell changes. Basket cells and Purkinje cells remained unchanged. In the Gasserian ganglion only a few nerve cells were changed but there was no capsular cell proliferation.

Summing up the observations there was a widespread neuronal change with eosinophilic homogenization and shrinkage of the cytoplasm and nuclear hyperchromasia which was most marked in the animals in the chronic experiment, particularly in those receiving high doses. This was less obvious in the subacute experiment but also to some extent present in the controls. Drop out of Purkinje cells and basket cells was definitely shown only in the chronic experiment. Neuronal changes together with capsular cell proliferation in the Gasserian ganglion was found in both experimental groups though in larger numbers only in the chronic experiment. Round cell infiltration around vessels and in the parenchyma occurred in all animals of the subacute and in one of the chronic experiments but not in the controls.

Discussion

From the available literature dealing with the toxicity of TRI it is necessary to discriminate very carefully between cases of acute poisoning with rather unspecific symptoms due to the general anaesthesia, and cases of prolonged poisoning where focal deleterious effects of TRI or its metabolites may become evident (ADAMS *et al.* 1951 BARTONÍČEK & SOUČEK 1959· PENNAROLA *et al.* 1966)

There is no doubt that among the toxic effects following chronic industrial exposure of humans, those involving the central nervous system are the most striking. Both localized neurological symptoms and diffuse psychosomatic symptoms in man have been reported. One of the first serious attempts to elucidate the toxic effect of TRI in humans was that by BORBÉLY (1946) and by AHLMARK & FORSSMAN (1951a & 1951b) The individual variation in sensitivity to the toxic effect seems to play an important role which can be based on the different detoxication capacity or rather on the enzymatic equipment of the individual in question (COOPER & FRIEDMAN 1958 RASKIN & SOKOLOFF 1968)

Functional derangements in humans, especially those involving the ol factory (JAMES 1963 TRENSE 1965), optic (FRANT & WESTENDORP 1950), oculomotor (TRENSE 1965) trigeminal (BUXTON & HAYWORD 1967 MITCHELL & PARSONS-SMITH 1969) facial and vestibular nerves (FRA *et al.* 1966) and the telencephalic cortex (GRANDJEAN *et al.* 1955 BARDODĚJ & VYSKOČIL 1956 MILBY 1968) and cerebellum (BORBÉLY 1946) have been reported. Damage of the facial and ulnar nerve trunks resulting in prolonged conduction time has also been observed (FELDMAN 1968) Trigeminal palsy visual field disturbances and attacks of vertigo have been described (MITCHELL & PARSONS-SMITH 1969) The relief of trigeminal neuralgia by TRI injection can be explained by the mechanism mentioned by BUXTON & HAYWORD (1967). Derangements within the psychic sphere in the shape of organic dementia (BORBÉLY 1946 TRENSE 1965) and other psychic disturbances (TODD 1954 HARENKO 1967) have been published.

Parenteral administration of TRI was used as it is easy and precise and enabled us to calculate the amount of TRI retained with rather high precision. We obtained good results with this technique in our previous studies both with carbon disulphide (MICHALOVÁ *et al.* 1959) and with TRI (BARTONÍČEK & SOUČEK 1959) Rabbits were chosen as experimental animals because our previous studies on metabolism, excretion and steady state of TRI were done on this species.

The widespread nerve cell changes observed in this study are of a rather unspecific type. They are not easily distinguished from artifacts but their predominance in the experimental animals argues against this interpretation as does the concomitant changes in acid phosphatase activity discussed below as well as the pictures of neuronophagia, indicating neuronal injury These changes are suggestive of anoxic-ischaemic nerve cell damage, or a direct toxic effect of TRI. A similar change has been reported by PENNAROLA *et al.* (1966). ADAMS *et al.* (1951), on the other hand were not able to find any histological changes in the central nervous system. One should, however point out, that the doses of TRI given to rabbits were probably essentially lower than in our experiment. The impression of an increased acid phospha-

tase activity in experimental animals, presumably due to aggregation and/or increase in the size and number of neuronal lysosomes, are in keeping with an anoxic or toxic nerve cell damage. It supports the finding of neuronal derangement. A markedly increased acid as well as alkaline phosphatase activity in the leucocytes of humans chronically exposed to TRI has recently been observed (FAIBORSKÁ 1969)

These changes may be of interest in the discussion of the genesis of the psychoorganic syndrome or organic dementia shown by TRI-intoxicated individuals. The drop out of Purkinje cells and basket cells may be part of this widespread neuronal damage or may reflect a more selective vulner ability It may be of interest with regard to the clinical picture of disturbed balance while standing and walking, giddiness and dizzines, that has been reported.

In this context the nerve cell changes in cranial nerve nuclei including the Gasserian ganglion are also of interest. Thus, they may assist in clarifying the disturbed function of the oculomotor trigeminal, facial and statoacustic nerves described in the literature on chronic TRI poisoning. We should point out the absence of any pathological changes in the peripheral parts of the olfactory optic and trigeminal nerve trunks as well as in the eye and hypophysis.

The present neuropathological changes are no doubt mild in view of the massive exposure and very extended duration of the experiment. The body weight of the rabbits in the subacute experiments, where the weekly parenteral loading was about six times as high as in the chronic experiment, decreased by 9 / to 14 / i. e. average of 12 / The control rabbits increased in weight by 10 / All rabbits in the subacute group at the end of the trial were very thin, feeble and seemed exhausted. Their muscle tone was markedly decreased. However they did not exhibit any palsies or sphincter insufficiency

The chronically intoxicated group where the exposure was of varying duration, increased in body weight from 8 / to 77 / average 47 / The control group increased in body weight by 18 / to 97 / average 44 /. Thus, in this group, the *loading with TRI was not large enough to break* down the natural tolerance of the rabbits as was the case in the former group.

The focal cellular infiltration is suggestive of an encephalitis. Such alterations however are not a rare finding in laboratory animals. Thus, the limitation here to the experimental animals may be incidental in this rather small material On the other hand, it may be correlated with the drug treatment either as a direct toxic effect or indirectly as an encephalitis enhanced by immunosuppression due to the action of TRI This cellular infiltration occurred in all animals of the subacute experiment i. e. the very massively intoxicated rabbits, and only in one chronically intoxicated rabbit.

It is of interest that BORBÉLY (1964) regarded the first stage of chronic industrial poisoning in humans as toxic encephalosis, the second stage as an organic psychosyndrome and the last stage as a toxic Korsakoff psychosis.

Chronic and relatively milder exposure, on the other hand, appears to demonstrate the damaging effect of TRI on the central nervous system better than by subacute intoxication. This is further indicated by differences between animals in the chronically exposed group the severity being roughly correlated with the duration of the exposure to TRI It may also mean that further prolongation of the exposure would be desirable in order to produce a clear pathomorphological picture.

Our studies in rabbits revealed moderate morphological changes. There are, in our opinion, several possible explanations for this and the relative lack of symptoms as compared to human intoxication which may be stated in the following order of decreasing probability:

1) The quantitatively different metabolism in rabbits and humans. Rabbits excrete about a half less trichloroacetic acid but about twenty times more trichloroethanol than humans when calculated for the same exposure to TRI and per kilogram body weight (BARTONÍČEK & SOUČEK 1959) A reasonable explanation for the less damaging effect in rabbits would be the much larger excretion of the very toxic agent trichloroethanol as compared with human subjects.

2) The unusually high species tolerance of rabbits to TRI. It is well known that the species tolerance or resistence against poisoning agents is extremely variable *in different animal species. We have never seen any pronounced* neurological syndrome in our previous trials with TRI in rabbits even when the histological picture of the parenchymal organs such as liver and kidney was grossly altered (BARTONÍČEK & SOUČEK 1959). It is of interest to compare the loading of the subacutely and chronically intoxicated animals with that of experimentally exposed humans (BARTONÍČEK 1962) Thus, the human volunteers retained 0 014 g the rabbits 1 0 g in the subacute exposure or 0.17 g in the chronic exposure per day and kg body weight. It thus follows, that the rabbits were exposed to approximately 75 times higher daily doses in the subacute exposure and approximately 13 times higher doses in the chronic exposure as compared with the single experimental exposure of human volunteers in a gas chamber

3) The toxicity ascribed to TRI is not caused by TRI as such or by its biological metabolites but by breakdown products formed before application *in vitro* (SMITH 1966) e. g. carbonyldichloride hexachlorbenzene or dichloroacetylene by stabilizers added (e g. triethylamine) (SMITH 1966) or by volatile heavy metal complexes contaminating TRI during its manufacture (e. g. Pb, Cu, Ni). The TRI used here, however according to the specification of the manufacturer was practically free from such metals.

REFERENCES

Adams, E. M., H. Spencer V R. Rowe & D. Irish. Vapor toxicity of trichloroethylene determined by experiments on laboratory animals. *Arch. Ind. Hyg. Occup Med.* 1951 3 469–481.

Ahlmark, A. & S. Forssman. The effect of trichlorethylene on the organism. *Acta physiol scand.* 1951a, 22, 326–339

Ahlmark, A. & S. Forssman. Evaluating trichloroethylene vapours by urinalysis for trichloroacetic acid. *Arch. Ind. Hyg* 1951b, 3, 386–398.

Bardoděj, Z. & J Vyskočil. The problem of trichloroethylene in occupational medicine, *Arch. Ind. Health* 1956, 13, 581 592.

Barka, T & P J Anderson. Histochemistry theory practice and bibliography Harper and Row Publ. Inc. N. 4 Evenstone and London 1963

Bartoníček, V Der Einfluss einiger Stoffe auf die Harnausscheidung von Trichloroethanol und Trichloressigsäure beim Kaninchen. *Arch. Gewerbepath. Gewerbehyg.* 1960, 18, 317–326.

Bartoníček, V Metabolism and excretion of trichloroethylene after inhalation by human subjects. *Brit J Ind Med.* 1962, 19 134–141.

Bartoníček, V The effect of some substances on the elimination of trichloroethylene metabolites. *Arch. int pharmacodyn. therap* 1963, 144 69–85

Bartoníček, V & B Souček: Der Metabolismus des Trichloroethylens beim Kaninchen. *Arch. Gewerbepath. Gewerbehyg* 1959 17 283–293.

Bartoníček, V & J Teisinger Effect of tetraethyl thiuramdisulphide (disulfiram) on metabolism of trichloroethylene in man. *Brit. J Ind Med.* 1962, 19 216–221.

Borbély F Erkennung und Behandlung der organischen Lösungsmittelvergiftungen. Medizinischer Verlag Hans Huber Bern, 1946.

Buxton, P H. & M. Hayword. Polyneuritis cranialis associated with industrial trichloroethylene poisoning. *J Neurol. Neurosurg Psychiat.* 1967 30 511–518.

Cooper J R. & P J Friedman. The enzymic oxidation of chloralhydrate to trichloroacetic acid. *Biochem. Pharmacol.* 1958, 1 76–82.

Feldman, R. G & R. F Mayer Studies of trichloroethylene intoxication in man. *Neurology* (Minn.) 1968, 18 309

Fra, L., G Gandiglio, A. Riccio & G. Sandigliano: Osservazione clinica e studio neurofisiologico di un caso di intossicazione cronica da tricloroetilene. *Med. lavoro* 1966, 57 606–613.

Frant, R. & J Westendorp Medical control on exposure of industrial workers to trichloroethylene. *Arch. Ind. Hyg Occup Med* 1950, 4 308–318.

Friborská, A. The phosphatases of peripheral white blood cells in workers exposed to trichloroethylene and perchloroethylene. *Brit. J Ind. Med.* 1969 26, 159–161.

Grandjean, E., R. Muenchinger V *Turrian*, P A. *Hass*, H. K. *Knöpfel* & H. Rosmund: Investigation into the effects of exposure to trichloroethylene in mechanical *engineering.Brit. J Ind. Med.* 1955 12, 131 146.

Harenko, K. Two peculiar instances of psychotic disturbance in trichloroethylene poisoning. *Acta neurol. scand.* 1967 43, Suppl. 31 139–140.

James, W R. L. Fatal addiction to trichloroethylene. *Brit J Ind Med.* 1963, 20, 47–49

Mahon, G S. Staining for myelin sheets in tissues embedded in paraffin. *Arch. Neurol. Psych.* 1937 38, 103–107

Michalová, C., V *Bartoníček* & V *Zástava. Organotoxische Wirkungen der* Schwefelkohlenstoff nach parenteralen Intoxikation von Kaninchen. *Arch. Gewerbepath. Gewerbehyg* 1959 16, 653–665

Milby T H. Chronic trichloroethylene intoxication. *J Occup Med.* 1968, 10, 252–254.

Mitchell, A. B. S. & G. B. Parsons-Smith. Trichloroethylene neuropathy. *Brit. Med. J.* 1969, 1, 422–423.

Pennarola, R., P. Lamanna & N. Castellino: Reperti istopatologici nell intossicazione sperimentale da tricloroetilene. *Folia med.* 1966, 49, 853–864.

Raskin, N. H. & R. Sokoloff. Brain alcohol dehydrogenase. *Science* 1968, 162, 131–132.

Smith, F. G. Trichloroethylene: A Review. *Brit. J. Industr. Med.* 1966, 23, 249–262.

Todd, J. Trichloroethylene poisoning with paranoid psychosis and lilliputian hallucinations. *Brit. Med. J.* 1954, 1, 439–440.

Tresse, E., Praktische Ergebnisse der Untersuchung von 546 Tri-Arbeitern. *Ztbl. Arbeitsmed.* 1965, 15, 546–548.

Acta pharmacol et toxicol 1970 28, 370–377

From the Department of Pharmacology
University of Copenhagen, Denmark

The Effect of d-Tubocurarine on the Kinetics of Decamethonium Uptake by Mouse Kidney Slices

By

Jan Holm

(Received March 12, 1970)

Abstract The effect of d-tubocurarine on the kinetics of the decamethonium uptake by mouse kidney slices was studied. Slices were incubated (1 hour) in a Krebs-Ringer bicarbonate medium (37° pH 7.4) in the presence of ^{14}C-decamethonium and d-tubocurarine. With increasing d-tubocurarine concentration the ^{14}C-slice-to-medium concentration ratio decreased towards a constant value (1.3) regardless of the decamethonium concentration used. The decamethonium uptake can thus be divided into two components, one which is sensitive to d-tubocurarine (curare-sensitive) and one which is not (curare-insensitive). The latter component increased proportionally with the external decamethonium concentration. The curare-sensitive component, which showed saturation with increasing decamethonium concentration, had a maximum capacity of 1100–1280 µM kg^{-1} h^{-1} and a half saturation concentration of 175–180 µM. The latter component was inhibited by d-tubocurarine with an inhibitor constant of 12 µM and the inhibition was in accordance with competitive antagonism. The result suggest that decamethonium is taken up by two distinct processes, one mediated by a carrier and one by a passive diffusion into the cells. Furthermore the carrier is common to both decamethonium and d-tubocurarine.

Key-words. Decamethonium compounds – tubocurarine – kinetics.

Recent *in vitro* studies (HOLM 1970) have shown that ^{14}C-decamethonium is concentrated in mouse kidney slices by a partly energy dependent process. It was further shown that the uptake consisted of at least two components, one that showed saturation with increasing decamethonium concentration in the medium and one that increased proportionally with the external decamethonium concentration. Both ^{3}H-decamethonium and ^{3}H-carbamoylcholine accumulate in slices of rat cerebral cortex (TAYLOR *et al* 1969). The uptake of ^{3}H-carbamoylcholine (CREESE & TAYLOR 1967) consisted of two components, a saturable one markedly inhibited by d-tubocurarine and a linear one very much less sensitive to the inhibitor The inhibitory action of

d-tubocurarine on the saturable part of the uptake was in accordance with competitive antagonism. Thus the question arose whether d-tubocurarine would inhibit the renal *in vitro* uptake of decamethonium in mice and partic ularly the saturable component of the uptake process.

The object of the present study* was to examine the effect of d-tubocurarine on the uptake of ^{14}C-decamethonium by mouse kidney slices. For this purpose the relationship between the ^{14}C-decamethonium uptake and the d-tubocurarine concentration was determined.

Methods

Materials.

^{14}C-methyl-decamethonium dibromide (specific activity 20.9 mci/mmol) was obtained from the Radiochemical Centre, Amersham, England.

Unlabelled decamethonium dibromide (syncurine®) and decamethonium diiodide were received from Burroughs Wellcome and Co. Unlabelled d-tubocurarine dichloride was supplied by Abbott, France and by A/S Alfred Benzon, Copenhagen (tubocuran®).

Experimental procedure

The method described in detail by HOLM (1970) was used. Three hundred and thirty-three male albino mice of a single strain (NMRI) ranging in weight from 28–32 g were decapitated and bled. Kidney slices were prepared with razor blade and ten to twelve slices (total wet weight 200 mg) from the same animal were placed in test tube, which contained 20 ml Krebs-Ringer bicarbonate solution with glucose (2 g/l). The test tubes were shaken for one hour at 37 (pH 7.4) and a 5 95 mixture of carbon dioxide-oxygen was bubbled through the medium. The slices were submerged in the salt solution 10 minutes before the addition of drugs. Approximately the same amount of radioactive material (0.2 µci) was used in each f the experiments and the desired concentration of decamethonium was always obtained by adding unlabelled carrier Unlabelled d-tubocurarine was added to the media just before ^{14}C-decamethonium.

Measurement of radioactivity

At the end f the incubation period the slices were separated from the media by filtration on cotton and weighed Tissue and medium samples were prepared and the radioactivity of each sample was measured by means of Packard Tri-Carb liquid scintillation spectrometer model 314 EX as previously described (HOLM 1970).

The results were expressed as slice-to-medium (S/M) concentration ratio of ^{14}C calculated as the counting rat per g slice (wet weight)/counting rat per ml medium. In some experiments the uptake of ^{14}C-decamethonium by the kidney slices (µmol per kg tissue) was calculated from the S/M ratio and the ^{14}C-decamethonium concentration of the medium (µmol per l medium).

Part of this study was presented at the XIII Scandinavian Congress of Physiology Göteborg 1969

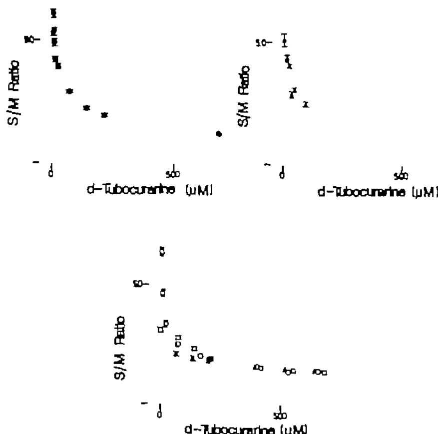


Fig. 1. Relationship between the slice-to-medium (S/M) concentration ratio of ^{14}C-decamethonium after incubation for one hour and the concentration (μM) of d-tubocurarine in the medium. The points represent mean values from 5–12 experiments and the vertical bars indicate S. E. M. The decamethonium concentration of the medium was 45 μM (●) in fig. 1a and 180 μM (▲) in fig. 1b. The following decamethonium concentrations were used in fig. 1c: 90 μM (□), 540 μM (○), 900 μM (△) and 1800 μM (■).

Results

The effect of d-tubocurarine on decamethonium uptake

In fig. 1 the slice-to-medium (S/M) concentration ratio of ^{14}C-decamethonium after incubation for one hour has been drawn as the function of the d-tubocurarine concentration (μM) in the medium. The results shown in

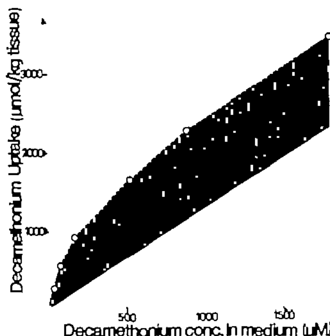


Fig. 2. The uptake of decamethonium (µmol/kg tissue) as a function of the decamethonium concentration in the medium. The upper curve (dotted), which represents the total decamethonium uptake, was recently published (HOLM 1970). Open circles represent mean values. The straight unbroken line represents the curare-insensitive uptake (calculated from the average value 1.3 and the decamethonium concentration in the medium; for details, see legend of fig. 1 and text). The hatched area in the figure represents the curare-sensitive uptake.

fig. 1a were obtained with 45 µM decamethonium in the medium. The results shown in fig. 1b were obtained with 180 µM decamethonium in the medium and those in fig. 1c with 90 µM, 540 µM, 900 µM and 1800 µM decamethonium respectively in the medium. It is seen that the decamethonium S/M ratio is considerably reduced even by low concentrations of d-tubocurarine (< 20 µM). With higher concentrations of d-tubocurarine the uptake (S/M ratio) is inhibited even more, but the sensitivity of the process to the inhibitor becomes considerably lower. Thus, the S/M ratio is seen to fall with increasing d-tubocurarine concentration towards a practically constant value around 1.3 (1.2–1.4) regardless of the decamethonium concentration used.

Based on the inhibitory effect of d-tubocurarine the decamethonium uptake can be divided into two components. One of these is curare-sensitive, while the other is practically insensitive to the inhibitor (curare-insensitive).

Decamethonium uptake as a function of the decamethonium concentration in the medium

The upper curve (dotted) in fig. 2, which shows the relationship between the total decamethonium uptake (µmol/kg tissue) after incubation for one hour and the concentration of decamethonium in the medium, was recently published (HOLM 1970) The decamethonium uptake seems to consist of at least two components, one that shows saturation with increasing decamethonium concentration in the medium and one that increases proportionally with the external concentration. The straight unbroken line represents the curare-insensitive uptake, calculated from the decamethonium concentration and the average concentration ratio of the curare-insensitive component 1.3 As far as can be seen, the curare-insensitive component is similar to the linear component of the total decamethonium uptake. The curare-sensitive component, represented by the hatched area in fig. 2, is consequently similar to the saturable part of the uptake

The concentration ratio for the curare-sensitive part of the decamethonium uptake (Sc/M) can be calculated as the difference between the concentration ratio for the whole uptake and the concentration ratio for the curare-insensitive component. In fig. 3 the reciprocal value of Sc/M has been plotted against the decamethonium concentration in the medium (M) in analogy to the method of DIXON & WEBB (1959) for the quantitative evaluation of enzymatic reactions. As the relation appears to be linear it is possible to calculate the maximum capacity of this uptake process as the reciprocal value of the slope of the line and to read the half saturation concentration as the negative value of the point at which the line intersects the abscissa. Thus calculated the maximum capacity (V) is found to be 1280 µM kg^{-1} h^{-1} and the half saturation concentration (K_m) to be 175 µM.

The action of d tubocurarine on the curare-sensitive part of the decamethonium uptake

The curare-sensitive uptake can be calculated from the Sc/M ratio (cf. legend and text to fig 3) and the ^{14}C-decamethonium concentration (µM) of the medium. In fig. 4 the reciprocal values of the curare-sensitive uptake (µmol/kg tissue) at two concentrations of ^{14}C-decamethonium (45 µM and 180 µM) have been plotted against the d-tubocurarine concentration (µM) of the medium in analogy to DIXON's method (1953) for the determination of enzyme inhibitor constants. The relation between the reciprocal values of the curare-sensitive uptake and the inhibitor concentration is, as far as can be seen, linear According to the criteria of DIXON (1953) the inhibition is competitive if the lines intersect at a point on the left of the vertical axis and it is possible to read the inhibitor constant (K_i) as the negative value of the corresponding point on the abscissa. Thus, the inhibitory effect of d-tubo-

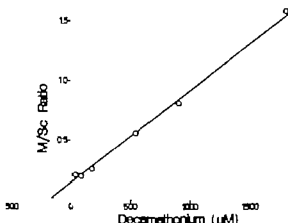


Fig. 3. Relationship between the reciprocal of curare-sensitive decamethonium uptake (M/Sc ratio) and the external concentration (μM) of decamethonium. Data from fig. 1 corrected for curare-insensitive uptake (Sc/M = S/M − 1.3 for details, see legend of fig. 1 and text).

curarine on the curare-sensitive part of the decamethonium uptake appears to be competitive and the inhibitor constant of d-tubocurarine is found to be 12 μM.

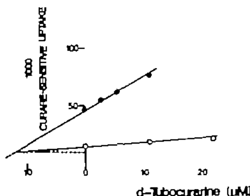


Fig. 4. Relationship between the reciprocal of curare-sensitive decamethonium uptake (μmol/kg) and the concentration (μM) of d-tubocurarine. Two concentrations of decamethonium were used, 45 μM (●) and 180 μM (○). Data from fig. 1a and 1b were corrected for curare-insensitive uptake (Sc/M = S/M − 1.3). The curare-sensitive uptake, expressed as μmol/kg tissue, was calculated from the Sc/M ratio and the decamethonium concentration of the medium (for details, see legends of fig. 1, 3 and text).

The half saturation concentration, K_m can also be determined by this method, as each of the lines intersects the abscissa at a value equal to $-K_i$ $(C/K_m + 1)$ where C is the decamethonium concentration (μM) The upper line (decamethonium concentration, 45 μM) intersects the abscissa at a point corresponding to − 15 μM and K_m is thus found to be 180 μM (the value 175 μM was found in fig 3 cf. text). Furthermore, it is possible to calculate the maximum capacity (V) of the curare-sensitive uptake, as the intersection point giving K_i lies at a height of a point (0.9) on the vertical axis corresponding to the reciprocal value of V Calculated by this method V is found to be 1100 μM kg^{-1} h^{-1} (the value 1280 μM kg^{-1} h^{-1} was found in fig. 3 cf. text).

Discussion

The present experiments show that the uptake of ^{14}C-decamethonium by mouse kidney slices is significantly reduced in the presence of d-tubocurarine. As shown, the decamethonium uptake can be divided into two processes. one that is very sensitive to d-tubocurarine (curare-sensitive) and one that operates even in the presence of very high d tubocurarine concentrations (curare-insensitive) The curare-sensitive process is saturable and the action of d-tubocurarine on this process can be described as competitive antagonism. The curare-insensitive process is directly proportional to the external decamethonium concentration and constitutes the major part of the uptake at high concentrations of decamethonium in the medium.

The above-described kinetics are most simply explained by assuming the presence of two distinct mechanisms for the uptake of decamethonium, one mediated by a carrier the other by a passive diffusion into the cells. The action of d tubocurarine on the curare-sensitive component of the uptake suggests that this agent competes with decamethonium for a common carrier Furthermore, the affinity of d-tubocurarine to this carrier should be very high as compared to that of decamethonium, as the half saturation concentration of decamethonium for the curare-sensitive component of the uptake exceeds the inhibitor constant of d tubocurarine by 14 times. CREESE & TAYLOR (1967), who studied the uptake of ^{3}H-carbamoylcholine in slices of rat brain, found that the half saturation concentration of carbamoylcholine for the curare-sensitive part of the uptake exceeded the inhibitor constant of d tubocurarine by about 13 times.

The curare-insensitive part of the decamethonium uptake exceeds the size of the sucrose space, as the slice-to-medium concentration ratio of ^{14}C-sucrose after one hour incubation is 0.54 (HOLM 1970). Thus, the curare-insensitive component cannot merely be ascribed to a simple distribution of decametho-

nium in the extracellular space. Part of the curare-insensitive component is therefore most likely due to a passive diffusion of decamethonium into the cells. The latter process might to some extent represent a passage of decamethonium through cell membranes damaged by the experimental technique.

Acknowledgements

The Packard Tri-Carb Scintillation spectrometer of the department used in the study was purchased with a grant given by the Danish State Research Foundation (A 5/65) Mr Bjarne Lauritzen gave technical assistance.

REFERENCES

Creese, R. & D. B. Taylor Entry of labelled carbachol in brain slices of the rat and the action of d-tubocurarine and strychnine. *J Pharmacol. Exp Ther* 1967 157 406–419

Dixon, M. The determination of enzyme inhibitor constants. *Biochem. J* 1953 55, 170–171.

Dixon, M. & E. C. Webb Enzymes 1959 1st. ed., 3rd impression, pp 19–22. London, Longman, Green and Co. Ltd

Holm, J The uptake of decamethonium and hexamethonium by slices of mouse kidney *Acta pharmacol. et toxicol.* 1970, 28, 192–202.

Taylor D. B., R. Creese & T.-C. Lu: The mode of action of morphine on the uptake of carbachol and decamethonium by slices of rat cerebral cortex. *J Pharmacol Exp Ther* 1969 165, 310–317

Acta pharmacol. et toxicol 1970 28, 378–390

From the Psychiatric Research Centre,
University of Göteborg, S:t Jörgen Hospital, and the Department of Pharmacology
University of Göteborg, Göteborg, Sweden

Drug Induced Changes in ³H-Catecholamine Accumulation after ³H Tyrosine

By

Torgny Persson

(Received April 27 1970)

Abstract· The accumulation of ³H DA and ³H NA in the caudate nucleus, remainder of the brain, spinal cord and heart was measured 15 minutes after ³H-tyrosine administered intravenously into rats pretreated with drugs. Apomorphine as well as stimulating DA receptors reduced ³H-DA when given shortly before ³H-tyrosine, probably due to a negative feed-back. When given 30 minutes or 3 hours previously ³H NA was increased, probably indicating the importance of DA neurone activity for NA activity Clonidine which stimulates NA receptors, did not significantly reduce ³H-NA as expected by feed-back. Pimozide, which blocks DA but apparently not NA receptors, not only increased ³H DA but also ³H NA. Haloperidol and chlorpromazine which block DA and NA receptors, only increased ³H DA. Thioridazine, however also increased ³H-NA. The complicated balance between the tendency for an increase of NA turnover by the direct feed-back mechanism, and the counteracting inhibition from a reduced stimulation from the DA neurones, seems to be a contributary factor LSD which stimulates the *5-HT* receptors increased the ³H-catecholamines both centrally and peripherally The effect of d-amphetamine was studied in some experiments, the most striking effect being an increase in ³H DA after an intravenous injection of the drug. Lithium carbonate increased ³H-NA and probably also ³H-DA. Phenoxybenzamine, which blocks NA receptors, increased ³H-NA via feed-back regulation.

Key-words: *Catecholamines – tyrosine – drugs.*

Most psychoactive drugs interfere with central monoaminergic transmission (see CARLSSON 1967) The neuroleptic *drugs, such as chlorpromazine* and haloperidol, exert little or no effect an brain catecholamine levels unless given in high doses in long-term experiments (FALCK *et al.* 1969) but will after *monoamine oxidase inhibition*, accelerate the accumulation of the 0-methylated metabolites (CARLSSON & LINDQVIST 1963), indicating an increased amine turnover Later studies of other catecholamine metabolites also indicated such effects (ANDÉN *et al.* 1964 SHARMAN 1966). More directly performed

turnover estimations by the tracer technique (GEY & PLETSCHER 1968 NYBÄCK *et al.* 1968), or with tyrosine hydroxylase inhibitors (SHARMAN 1966 CORRODI *et al.* 1967a ANDÉN *et al* 1967a & 1970a), showed that the neuroleptics increased dopamine (DA) and to a lesser extent noradrenaline (NA) turnover Evidence is accumulating that drugs blocking or stimulating monoamine receptors induce changes in nerve activity and consequently in amine turnover via feed-back mechanisms (see ANDÉN *et al.* 1969) The mechanisms may be still more complicated, since there appears to be a functional interaction between e. g. DA and NA neurones (PERSSON & WALDECK 1970a & b). An impaired DA transmission mechanism seems to retard NA turnover whilst stimulation of DA receptors increases NA turnover In this investigation drugs which act on central monoaminergic mechanisms have been screened for their effects on catecholamine formation. Some of these drugs are already of great importance in psychiatry The drugs studied have little or no effect on catecholamine levels but pronounced effects on turnover (see ANDÉN *et al* 1969). With the tracer technique used, the possible interference *of synthesis inhibitors* (*see* PERSSON & WALDECK *1970a & b*) on the transmission and turnover is avoided. The methods for turnover estimations give no absolute values for turnover (see PERSSON & WALDECK 1970c) but useful conclusions can be drawn from the relative changes. In this study the influence of different drugs on the conversion of L 3 5-^{3}H tyrosine to labelled catecholamines, has been examined in different regions of the central nervous system and in the heart of rats. The accumulation of the labelled catecholamines should be regarded as net accumulation and not as an absolute measure of synthesis (see NEFF *et al.* 1970 for discussion of these problems).

Material and methods

Male albino rats weighing 150–200 grams were used. The experiments were carried out at an ambient temperature of + 30 Rectal temperatures were regularly checked to ascertain the absence of hypo- or hyperthermia. L 3, 5-^{3}H-tyrosin with specific activity of 35–54 ci/mmole, obtained from The Radiochemical Centre, Amersham, England, was given intravenously in dose of 5 μg/kg. The purity of the precursor was checked by radio-paper chromatography The drugs were given intraperitoneally except for two experiments with *d*-amphetamine. The drugs used, doses expressed as the salts and intervals at which they are given are shown in tables 1 and 2. The animals were killed by inhaling chloroform 15 minutes after the injection of ^{3}H-tyrosine. The following tissues, in each experiment pooled from two rats, were removed for analysis: The caudate nucleus, the remainder of th brain, the spinal cord and the heart (PERSSON 1969). *Separation of ^{3}H-NA and ^{3}H DA, was performed as described* previously (PERSSON & WALDECK 1968). When calculating the ^{3}H-catecholamine levels, the loss of one atom of tritium in the transformation of ^{3}H-tyrosine to ^{3}H DOPA

was accounted for. Due to the NIH-shift, i. e. intramolecular migration of the tritium (for review see DALY 1969) the net loss is actually less than 1 atom. The statistical analyses were performed by the use of variance analysis and Dunnett's tables for multiple comparisons with a control (DUNNETT 1964).

During the experiments the drugs were checked in order to ascertain whether they elicited the expected pharmacological effects on the animals and whether the effects were maintained during the whole experiment (i. e. gnawing and stereotypic behaviour after apomorphine, piloerection and eye ball protrusion after clonidine, sedation after phenoxybenzamine and the neuroleptic drugs).

In the phenoxybenzamine experiments (see tables 1 and 2) the drug was given either completely dissolved by warming to 40° or as a suspension, since it has been observed (ANDÉN personal communication) that the drug loses a considerable part of its activity on warming.

Results

The experimental data with the statistical results are shown in tables 1 and 2. The results are summarised in table 3 where the ^{3}H-catecholamine levels after the different drugs are expressed as per cents of the controls.

The experiments were run in two series. They are presented separately because the control values of ^{3}H DA in the two series differed from each other ($P < 0.001$ for ^{3}H DA in the caudate nucleus and $P < 0.005$ for ^{3}H-DA in the remainder of the brain). The ^{3}H-NA levels also differed between the two series. Possible causes of these differences are seasonal variations in the animals and analytical variations.

In the following only the principal changes in the accumulation of the ^{3}H-catecholamines after the different drugs are described. For ^{3}H DA alone, the levels in the caudate nucleus are described. The ^{3}H DA in the remainder of the brain is not only derived from DA neurones but also from NA neurones, where ^{3}H DA occurs as an intermediate metabolite in the ^{3}H-NA synthesis. At intervals of 15 minutes after ^{3}H-tyrosine, the amounts of ^{3}H-DA and ^{3}H NA were similar in the NA regions (PERSSON 1969).

Apomorphine reduced the accumulation of ^{3}H DA when given 15 minutes before ^{3}H tyrosine. The ^{3}H-NA levels were unchanged. In the 30 minutes interval no significant effect was found on the accumulation of ^{3}H-DA, but the drug increased the accumulation of ^{3}H-NA in the central nervous system. Apomorphine had the same effect when given twice i. e. 3 and 1.5 hours before ^{3}H-tyrosine.

The only effect of *pimozide* when given in a dose of 0.5 mg/kg 30 minutes or 3 hours before the ^{3}H-tyrosine was to increase the accumulation of ^{3}H-DA in the caudate nucleus. In a dose of 10 mg/kg given 30 minutes before the precursor the drug increased ^{3}H DA in the caudate nucleus and ^{3}H-NA in the remainder of the brain.

Table 1

Regional accumulation of ^{3}H-DA and ^{3}H NA after ^{3}H-tyrosine in rats pretreated with different drugs influencing central monoaminergic mechanisms. The ^{3}H-catecholamines were determined 15 minutes after an intravenous injection of 5 µg/kg of ^{3}H-tyrosine. The drugs were given as listed in the table. The mean levels ± S. E. M. are shown. The levels are expressed as fmoles/g tissue, 1 fmole = 1 femtomol = 10^{-15} moles. Statistical analysis was performed with use of variance analysis and Dunnett's tables. N = number of determinations. NS = not significant. The drugs were given intraperitoneally except for d-amphetamine, which was given intravenously

Drugs	N	Dose mg/kg	Interval drug ^{3}H-tyrosine min.	^{3}H DA in caudate nucleus	^{3}H-DA in remainder of the brain	^{3}H-NA in remainder of the brain	^{3}H NA in spinal cord	^{3}H NA in heart
Controls	4	–		435 ± 17	31 ± 2	19 ± 1	16 ± 1	20 ± 2
Apomorphine	3	25	30	277 ± 16 NS	22 ± 4 NS	32 ± 1 P < 0.01	29 ± 4 P < 0.05	4 ± 5 NS
Pimozide	2	0.5	30	700 ± 92 P < 0.05	43 ± 2 NS	23 ± 3 NS	15 ± 1 NS	16 ± 0 NS
Pimozide	2	10	30	2178 ± 96 P < 0.01	66 ± 24 P < 0.01	38 ± 14 P < 0.01	–	17 ± 3 NS
Haloperidol	3	0.5	30	1042 ± 108 P < 0.01	47 ± 3 NS	16 ± 2 NS	–	22 ± 1 NS
Haloperidol	4	2.5	30	670 ± 101 P < 0.01	49 ± 9 P < 0.05	18 ± 1 NS	19 ± 2 NS	9 ± 1 NS
Thioridazine	3	50	30	1339 ± 2 P < 0.01	64 ± 10 P < 0.01	38 ± 4 P < 0.01	38 ± 4 P < 0.01	26 ± 3 NS
Chlorpromazine	3	50	30	1219 ± 48 P < 0.01	73 ± 8 P < 0.01	24 ± 3 NS	20 ± 3 NS	20 ± 3 NS
Clonidine	4	3	15	659 ± 57 P < 0.01	45 ± 7 NS	14 ± 1 NS	12 ± 2 NS	24 ± 6 NS
Phenoxybenzamine (as solution)	4	20	30	594 ± 66 NS	44 ± 6 NS	34 ± 3 P < 0.01	20 ± 1 NS	50 ± 7 P < 0.01
d-Amphetamine	2	0.5	5	656 ± 225 NS		20 ± 5 NS	16 ± 4 NS	10 ± 6 NS
d-Amphetamine	5	1.0	5	917 ± 59 P < 0.01	35 ± 2 NS	18 ± 2 NS	15 ± 2 NS	18 ± 6 NS

Table 2

Regional accumulation of ^{3}H-DA and ^{3}H-NA after ^{3}H-tyrosine in rats pretreated with various drugs. For details see table 1.

Drugs	N	Dose mg/kg	Interval drug ^{3}H-tyrosine	^{3}H-DA in caudate nucleus		^{3}H-DA in remainder of the brain		^{3}H NA in remainder of the brain		^{3}H NA in spinal cord		^{3}H-NA in heart	
Controls	19	–	–	330 ± 15		23 ± 1		14 ± 1		12 ± 1		16 ± 3	
Apomorphine	6	25	15 min.	54 ± 6	$P < 0.01$	6 ± 1	$P < 0.01$	9 ± 1	NS	12 ± 3	NS	26 ± 3	NS
Apomorphine	4	25, 25	3 1.5 hrs.	353 ± 23	NS	24 ± 2	NS	26 ± 4	$P < 0.01$	24 ± 4	$P < 0.01$	18 ± 4	NS
Pimozide	4	0.5	3 hrs	706 ± 44	$P < 0.01$	40 ± 1	NS	18 ± 1	NS	18 ± 2	NS	16 ± 2	NS
Haloperidol	4	1	3 hrs	644 ± 118	$P < 0.01$	30 ± 2	$P < 0.01$	13 ± 1	NS	12 ± 1	NS	19 ± 10	NS
Chlorpromazine	4	50	3 hrs	912 ± 58	$P < 0.01$	34 ± 3	$P < 0.01$	14 ± 1	NS	10 ± 1	NS	8 ± 1	NS
Clonidine	4	1	15 min.	543 ± 46	$P < 0.01$	36 ± 1	$P < 0.01$	12 ± 1	NS	11 ± 1	NS	8 ± 1	NS
Clonidine	4	1	2 hrs	434 ± 22	NS	32 ± 1	$P < 0.05$	8 ± 1	NS	10 ± 3	NS	14 ± 1	NS
Clonidine	4	3	3 hrs	358 ± 21	NS	19 ± 3	NS	8 ± 1	NS	6 ± 1	NS	9 ± 1	NS
Phenoxybenzamine (suspension)	4	20	30 min.	494 ± 52	$P < 0.05$	36 ± 3	$P < 0.01$	26 ± 3	$P < 0.01$	22 ± 2	$P < 0.05$	35 ± 4	$P < 0.01$
Phenoxybenzamine (solution)	3	20	30 min.	400 ± 84	NS	23 ± 6	NS	21 ± 2	$P < 0.05$	29 ± 3	$P < 0.01$	29 ± 4	NS
Phenoxybenzamine (suspension)	4	20	2 hrs	352 ± 39	NS	29 ± 3	NS	32 ± 3	$P < 0.01$	33 ± 5	$P < 0.01$	37 ± 3	$P < 0.01$
Phenoxybenzamine (suspension)	4	25	3 hrs	381 ± 17	NS	32 ± 3	$P < 0.05$	27 ± 1	$P < 0.01$	33 ± 7	$P < 0.01$	37 ± 5	$P < 0.01$
d-Amphetamine	4	1	30 min.	402 ± 50	NS	22 ± 1	NS	13 ± 1	NS	10 ± 1	NS	8 ± 1	NS
d-Amphetamine	4	1, 0.5	3, 1 hrs	370 ± 33	NS	12 ± 1	$P < 0.01$	15 ± 1	NS	15 ± 2	NS	9 ± 0	NS
LSD-25	2	2	15 min.	929 ± 166	$P < 0.01$	22 ± 0	NS	41 ± 8	$P < 0.01$	41 ± 8	$P < 0.01$	45 ± 0	$P < 0.01$
N, N,-dimethyl-tryptamine	4	50	3 hrs	313 ± 65	NS	21 ± 2	NS	16 ± 2	NS	16 ± 0	NS	10 ± 2	NS
Lithium carbonate	4	150	4 hrs	455 ± 78	NS	32 ± 3	$P < 0.05$	21 ± 4	$P < 0.05$	16 ± 0	NS	17 ± 3	NS

Table 3

Drug-induced changes in regional ^{3}H-catecholamine levels following ^{3}H-tyrosine administration. The tabl is a summary of data from tables 1 and 2 and shows the mean levels expressed in per cent of the controls. The statistically significant changes are indicated with asterics (* $P < 0.05$ ** $P < 0.01$). If not otherwise stated, the drugs were given intraperitoneally

Drug	Dose mg/kg	Interval drug ^{3}H tyrosine	^{3}H-DA caudate nucleus	^{3}H DA remainder of the brain	^{3}H-NA remainder of the brain	^{3}H NA spinal cord	^{3}H-NA heart
Apo-morphine	25	15 min.	16	26	64	100	163
	25	30 min.	64	71	178**	181	120
	25, 25†	3,1 hrs†	107	104	186**	200*	113
Pimozide	0.5	30 min.	161	139	128	94	80
	10	30 min.	500*	213**	217**	–	85
	0.5	3 hrs	214**	174**	129	150	100
Haloperidol	0.5	30 min.	240*	152	89	–	110
	2.5	30 min.	154	158	100	119	45
	1	3 hrs	195	135	93	100	119
Chlor promazine	50	30 min.	280**	236	135	125	100
	50	3 hrs	276**	148*	100	83	50
Thioridazine	50	30 min.	306**	207*	211	238*	130
Clonidine	3	15 min.	152**	145	78	75	120
	1	15 min.	165*	157	86	92	50
	1	2 hrs	138	139*	57	83	88
	3	3 hrs	109	83	57	50	56
Phenoxy-benzamine							
solution	20	30 min.	137	142	189**	125	250*
suspension	20	30 min.	150*	157**	186**	183	219**
solution	20	30 min.	121	100	150*	242**	181
suspension	20	2 hrs	107	126	229**	275	231**
suspension	25	3 hrs	116	139*	193	275**	231
d-Amphetamine	0.5 i.v	5 min.	157	–	111	100	50
	1.0 i.	5 min.	211	113	100	94	90
	1.0	30 min.	122	96	93	83	50
	1.0,0 5†	3,1 hrs†	112	52**	107	125	56
LSD-25	2	15 min.	282	96	293	342**	281**
N,N-dimethyl-tryptamine	50	3 hrs	95	91	114	133	39
Lithium carbonate	150	4 hrs	138	139*	150*	133	65

†) The doses were repeated, i. e. for apomorphine 25 mg/kg 3 and 1 hr before ^{3}H-tyrosine and for d-amphetamine 1.0 and 0.5 mg/kg 3 and 1 hr before ^{3}H-tyrosine.

Haloperidol 0.5 or 2.5 mg/kg given 30 minutes before the precursor caused an increase in the accumulation of ^{3}H DA which was probably less than after the higher dose. Haloperidol 1 mg/kg 3 hours before the precursor also increased ^{3}H DA. No changes in the ^{3}H NA levels occurred.

Chlorpromazine had essentially the same effects as haloperidol.

Thioridazine increased ^{3}H-DA and ^{3}H-NA in the central nervous system. In the heart ^{3}H NA was not significantly changed.

Clonidine in doses of 1 and 3 mg/kg given 15 minutes before the precursor caused an increase in the accumulation of ^{3}H DA. In a dose of 1 mg/kg given 2 hours before ^{3}H-tyrosine, ^{3}H DA in the caudate nucleus and in the remainder of the brain was increased, in the latter tissue significantly With 3 mg/kg 3 hours before the precursor no changes in the ^{3}H DA levels were apparent. Clonidine in the given doses and intervals had no significant effect on the accumulation of ^{3}H NA.

Phenoxybenzamine (bensylytum NFN) was given as solution or suspension (see Materials and methods) When 20 mg/kg was given as a suspension 30 minutes before ^{3}H-tyrosine, the accumulation of ^{3}H-DA and ^{3}H-NA was increased both centrally and peripherally (table 2) With the same dose of phenoxybenzamine given as a solution, changes in the accumulation of ^{3}H-NA were only apparent centrally in one of the experimental series (table 2) but also in the heart in the other series (table 1) When the drug had acted for 2 hours before the injection of ^{3}H tyrosine, the accumulation of ^{3}H-NA was increased both centrally and peripherally The accumulation of ^{3}H-DA was not significantly affected. When 25 mg/kg of phenoxybenzamine was given 3 hours before the injection of ^{3}H-tyrosine, the same changes as in the abovementioned experiment occurred together with an increase in ^{3}H DA in the brain tissue except in the caudate nucleus.

D-amphetamine (as bitartrate) 0.5 mg/kg given intravenously 5 minutes before ^{3}H-tyrosine, caused no changes in the accumulation of ^{3}H-catecholamines but after 1 mg/kg the ^{3}H DA was increased. With 1 mg/kg given intraperitoneally 30 minutes before the precursor the accumulation of ^{3}H DA in the caudate nucleus was reduced.

LSD-25 2 mg/kg 15 minutes before the precursor increased the accumulation of both ^{3}H-catecholamines centrally and of ^{3}H NA in the heart.

N,N-dimethyltryptamine 50 mg/kg given 3 hours before the precursor did not cause any significant changes

Lithium carbonate 150 mg/kg given 4 hours before the precursor caused an increase (non-significant) of ^{3}H DA in the caudate nucleus and a significant increase in the remainder of the brain. ^{3}H NA was increased in the brain except in the caudate nucleus. The drug caused diarrhoea, slight tremor and motor unrest in the animals.

The levels of ^{3}H DA in the spinal cord and the heart (i. e. ^{3}H-DA as the

intermediate product in the synthesis of ^{3}H-NA in these organs), were followed as shown in the experiments shown in table 1 but no significant changes occurred.

Discussion

In this study L 3,5-^{3}H-tyrosine was used. Due to this labelling the conversion of the precursor to ^{3}H DOPA may be affected by a tritium rate effect. However the *in vitro* studies of tyrosinase by POMERANTZ (1966) do not suggest such an effect.

The drugs given may affect tyrosine metabolism outside the catecholamine neurones, in turn changing the specific activity of the precursor used for catecholamine synthesis. Tyrosine aminotransferase activity in rats is increased by phentolamine (GOVIER & LOVENBERG 1969) reserpine, guanethidine and α-methyl-para-tyrosine but not by phenoxybenzamine (GOVIER *et al.* 1969). An increased enzyme activity was accompanied by higher plasma corticosteroid concentrations. The enzyme changes, however had a lag period of one hour This mechanism is therefore unlikely to have affected the experiments in table 1 and those a short duration in table 2.

In turnover studies with tyrosine hydroxylase inhibitors (see ANDÉN *et al.* 1969) the rate of depletion of the catecholamines decreased when the receptors were stimulated and increased when the receptors were blocked. In the present investigation apomorphine was used to stimulate the central DA receptors and clonidine to stimulate the NA receptors. After apomorphine, the apparent DA turnover was reduced when the drug was given 30 minutes before the catecholamine precursor This was not the case at longer time intervals, in spite of obvious pharmacological effects such as gnawing. In experiments with tyrosine hydroxylase inhibitors, rather small doses of apomorphine reduced the rate of disappearance of DA (ANDÉN *et al* 1967b BUTCHER & ANDÉN 1969) The reasons for this discrepancy are unknown. In the present study the effects were measured for only 15 minutes. When apomorphine had acted for 30 minutes or longer an increased NA synthesis appeared as indicated by the accumulation of ^{3}H-NA. This finding agrees with the assumed functional interrelationship between DA and NA neurones (PERSSON & WALDECK 1970a and b), i. e. stimulation of the DA receptors causes an increase in the activity of the NA neurones and, in turn an increase of NA turnover That no indications of an increased NA turnover after apomorphine have been obtained in the studies with the tyrosine hydroxylase inhibitor (ANDÉN *et al.* 1967b) may be due to the short interval available for the synthesis inhibition, i. e. one hour Clonidine, a drug whi

stimulates the NA receptors (see ANDÉN *et al.* 1970b), failed to reduce the accumulation of ³H NA significantly in spite of evident pharmacological effects. However a tendency to reduced values was observed. The decrease in NA levels induced by inhibition of DA-β-hydroxylase in mice (PERSSON & WALDECK 1970b) was abolished by the doses of clonidine used in the present study The discrepancies indicate the need for using the methods in such a way that they are comparative and complimentary to one another With the shorter intervals between the clonidine and the ³H-tyrosine injections, the accumulation of ³H DA was increased. Possibly the marked stimulation of the NA receptors elicited an increased activity in the DA neurones. Catecholamine turnover studies with synthesis inhibitors and clonidine (ANDÉN *et al* 1970b PERSSON & WALDECK 1970b) apparently demonstrated a reduced DA turnover but here the experiments lasted longer and hence the nerve activity may have changed.

The 5-hydroxytryptamine (5-HT) receptor stimulating agent LSD-25 (ANDÉN *et al.* 1968) caused an increase in the accumulation of ³H-catecholamines both centrally and peripherally (in the heart) suggesting that stimulation of the 5-HT receptors secondarily increased the activity of catecholamine neurones The findings is analogous to that in synthesis inhibitor studies (ANDÉN *et al.* 1970c) In order to achieve a longer period of 5-HT receptor stimulation N N-dimetyltryptamine was used (because of shortage of the supplies of LSD-25) but no changes were observed. Here too, there appears to be a discrepancy between the results obtained with synthesis inhibitors and the ³H-tyrosine studies (c. f. ANDÉN *et al.* 1970c) One possible explanation is that the drug did not have a sufficiently strong action when the labelled precursor was given. The problem requires further studies using different doses and time intervals.

Amphetamine, an indirectly acting sympathomimetic amine was screened for its effects on the catecholamine turnover The drug has been shown to require a functioning mechanism of catecholamine synthesis for its central action (WEISSMAN & KOE 1965 WEISSMAN *et al.* 1966 HANSON 1967 JÖNSSON *et al.* 1969) With 1 mg/kg of d-amphetamine given intravenously 5 minutes before the precursor more ³H DA was accumulated but no changes occurred in the ³H NA levels. In the experiments where the drug was given 3 hours before the injection of ³H-tyrosine, ³H DA decreased in the brain tissue except in the caudate nucleus. These findings are difficult to interpret. In order to obtain an effect by amphetamine on NA turnover CORRODI *et al* (1967b) had to use large doses (10–15 mg/kg) and suggested, that the feedback regulation may counteract turnover changes due to amphetamine.

The DA receptors can be blocked with pimozide, a new neuroleptic drug apparently devoid of NA receptor blocking effects (see ANDÉN *et al.* 1970a). A considerable increase in the accumulated ³H DA indicated that the drug

elicited, via the feed-back regulation a significant increase in the turnover of DA. According to the discussed interrelationship between the central DA and NA neurones, pimozide should decrease the accumulation of ^{3}H NA but in fact it had the opposite effect though only in large doses. The studies with synthesis inhibitors (ANDÉN *et al.* 1970a) also indicated an increased turnover of NA. The possibility should be considered that pimozide in large doses may be capable of blocking central NA receptors.

The well-known neuroleptic drugs haloperidol, chlorpromazine and thioridazine block DA and NA receptors to varying extents (see ANDÉN *et al.* 1970a). The accumulation of ^{3}H-DA was increased, due to the blockade of the DA receptors, by these drugs. Only thioridazine increased the accumulation of ^{3}H-NA. That haloperidol and chlorpromazine did not increase the turnover of NA in this investigation, may be due to the simultaneous blockade of the DA receptors, which in turn prevented an increased NA neurone activity *via* the DA neurones. For thioridazine, the range between the doses necessary for the blockade of DA and NA receptors was found to be smaller than for the other two neuroleptics, whilst the doses necessary to obtain total receptor blockade were higher than for the other two It may be assumed therefore, that thioridazine only partially blocked the DA receptors, thus allowing an increase in the NA turnover due to the feed-back mechanism. Due to the complicated balance, the NA turnover may be affected by the neuroleptic drugs only at certain dose intervals (see ANDÉN *et al.* 1970a)

In this investigation, haloperidol and chlorpromazine increased only the accumulation of ^{3}H DA, which is in agreement with similar studies (NYBÄCK *et al.* 1968 GEY & PLETSCHER 1968). On the other hand, when the disappearance of labelled DA and NA, formed from a labelled precursor was followed after haloperidol, indications were obtained of an increased NA turnover (NYBÄCK *et al.* 1968) The reasons for these discrepancies are essentially unknown (vide infra)

After blockade of the NA receptors, induced by phenoxybenzamine, the expected increase in ^{3}H-NA occurred, centrally as well as peripherally There are many reports of an increased NA turnover after phenoxybenzamine (ANDÉN *et al* 1967a, BOULLIN *et al* 1967). Of the other drugs, only LSD-25 had an effect on the accumulation of ^{3}H NA in the heart.

Finally lithium carbonate was screened for possible actions on catecholamine turnover More ^{3}H-NA and ^{3}H DA accumulated in the brain tissue except in the caudate nucleus although a larger experimental material would probably also have revealed a significant increase in the accumulation of ^{3}H DA in the caudate nucleus. After tyrosine hydroxylase inhibition lithium accelerated the rate of disappearance of NA in brain (CORRODI *et al* 1967c). It is not known in what way lithium affects the turnover of

catecholamines It may act either indirectly or directly via changes in the neurone activity

Obvious discrepancies have thus been found when the results of these synthesis estimations are compared with results obtained with different methods used for turnover estimations. Some of the discrepancies may be ascribed to the differences in the doses and the time intervals of the drugs studied. There are, however also obvious methodological differences. Some of these have already been discussed but others have to be mentioned. Although the accumulation of labelled NA during the infusion of labelled tyrosine has been shown to be dependant on the nerve impulse flow the accumulation probably does not represent the whole synthesis (SEDVALL *et al.* 1968). Many different experimental studies indicate the existence of different pools of amine stores which are more or less available for release. KOPIN *et al.* (1968) have found evidence *in vitro* that more of the released NA derives from new synthesis than from the stores. The accumulated amines after injection or infusion of labelled tyrosine therefore probably represents only a part of the synthesis, the other part being lost during release. This mechanism may be responsible for some of the above mentioned differences.

Acknowledgements

This research was supported by grants from Göteborgs Läkaresällskap Medical Faculty of Göteborg, The Swedish Medical Research Council (B70-14X 155-06B) and Göteborgs and Bohus Läns Landsting.

The skilful assistance of Mrs. Lena Löfberg is gratefully acknowledged.

The following companies are thanked for generous gifts of drugs. C. H. Boehringer Sohn, Ingelheim (clonidine), Janssen Pharmaceutica, Beerse (pimozide) Leo Hälsingborg (chlorpromazine HCl, haloperidol and phenoxybenzamine), Regis, Chicago (N N-dimethyltryptamine) and Sandoz, Basle (apomorphine HCl, LSD-25 and thioridazine 2 HCl)

REFERENCES

Andén, N E., B. E. Roos & B. Werdinius: Effects of chlorpromazine, haloperidol and reserpine on the levels of phenolic acids in rabbit corpus striatum. *Life Sci.* 1964, 3, 149–158.

Andén, N E., H. Corrodi, K. Fuxe & T Hökfelt. Increased impulse flow in bulbo-spinal noradrenaline neurons produced by catecholamine receptor blocking agents. *European J Pharmacol.* 1967a, 2, 59–64.

Andén, N.-E., A. Rubenson, K. Fuxe & T Hökfelt: Evidence for dopamine receptor stimulation by apomorphine. *J Pharm. Pharmacol.* 1967b, 19 627–629

Andén, N. E., H. Corrodi, K. Fuxe & T Hökfelt: Evidence for central 5-hydroxytryptamine receptor stimulation by lysergic acid diethylamide. *Brit J Pharmacol.* 1968, 34, 1–7

Andén, N.-E., H. Corrodi & K. Fuxe: Turnover studies using synthesis inhibition. In. *Metabolism of amines in the brain* Symposium of the British and Scandinavian Pharmacological Societies, Edinburgh, July 1969 Ed. G Hopper MacMillan, London, 1969 pp. 38–47

Andén, N.-E., L. L. Butcher H. Corrodi, K. Fuxe & U Ungerstedt: Receptor activity and turnover rate of dopamine and noradrenaline after neuroleptics. *European J Pharmacol.* 1970a, in press.

Andén, N.-E., H. Corrodi, K. Fuxe, B. Hökfelt, T Hökfelt, C. Rydin & T Svensson. Evidence for a central noradrenaline receptor stimulation by clonidine. Lif Sci. 1970b, 9 513–523.

Andén, N.-E., H. Corrodi & K. Fuxe: Hallucinogenic drugs of the indolealkylamine type and central monoamine neurons. *J Pharmacol. exp Ther* 1970c, in press.

Boullin, D. J E. Costa & B. B. Brodie: Evidence that blockade of drenergic receptors causes overflow of norepinephrine in cats colon after nerve stimulation. *J Pharmacol. exp. Ther* 1967 157 125–134

Butcher L. L. & N. E. Andén: Effects of apomorphine and amphetamine on schedule-controlled behaviour: Reversal of tetrabenazine suppression and dopaminergic correlates. *European J Pharmacol.* 1969 6, 255–264

Carlsson, A. Basic actions of psychoactive drugs. *Int J Neurol.* 1967 6, 27–45.

Carlsson, A. & M. Lindqvist: Effect of chlorpromazine or haloperidol on formation of 3-methoxytyramine and normetanephrine in mouse brain. *Acta pharmacol et toxicol.* 1963, 20 140–144

Corrodi, H., K. Fuxe & T Hökfelt: The effect of neuroleptics on the activity of central catecholamine neurones. *Lif Sci.* 1967a, 6, 767–774.

Corrodi, H., K. Fuxe & T Hökfelt: The effect of some psychoactive drugs on central monoamine neurones. *European J Pharmacol* 1967b 1, 363–368.

Corrodi, H., K. Fuxe, T Hökfelt & M. Schou: The effect of lithium on cerebral monoamine neurons. *Psychopharmacologia* (Berl.) 1967c, 11 345–353.

Daly J The hydroxylation-induced migration of tritium. Theoretical aspects. *Atomlight*, N. E. N. 1969 No. 8, 1–7

Dunnett, C. W New tables for multiple comparisons with a control. *Biometrics* 1964 20 482–491.

Falck, B., L. Nordgren & E. Rosengren Effect of haloperidol on monoamines in the brain and heart. *Int. J Neuropharmac.* 1969 8, 631–634

Gey K.-F & A. Pletscher: Acceleration of turnover of ^{14}C-catecholamines in rat brain by chlorpromazine. Experientia 1968, 24, 335–336.

Gowler W C. & W Lovenberg: Induction of tyrosine aminotransferase by phentolamine. *Biochem. Pharmacol.* 1969 18, 2667 2672.

Gowler, W C., W Lovenberg & A. Sjoerdsma. Studies on the role of catecholamines as regulators of tyrosine aminotransferase. *Biochem. Pharmacol.* 1969 18, 2661–2666.

Hanson, L. C. F Evidence that the central action of (+)-amphetamine is mediated via catecholamines. *Psychopharmacologia* (Berl.) 1967 10 289–297

Jönsson, L.-E., L. M. Gunne & E. Änggård. Effects of α-methyltyrosine in amphetamine-dependent subjects. *Pharmacologia Clin.* 1969 2, 27–29.

Kopin, I. J G. R. Breese, K. R. Krauss & V K. Weise: Selective release f norepinephrine from the cat spleen during sympathetic nerve stimulation. *J Pharmacol. exp Ther* 1968, 161 271 278.

Neff, N H., S. H. Ngai, C. T Wang & E. Costa. Calculation of the rate of catecholamine synthesis from the conversion of tyrosine-^{14}C to catecholamines. Effect of adrenal medullation on synthesis rates. *Mol. Pharmacol.* 1970, 6, 90–99

Nybäck, H., Z. Borzecki & G Sedvall. Accumulation and disappearance of catechol amines formed from tyrosine-^{14}C in mouse brain. Effect of some psychotropic drugs. *European J Pharmacol.* 1968, 4 395–403.

Persson, T Central and peripheral catecholamine turnover studied by means of ^{3}H DOPA and ^{3}H-tyrosine. *Acta pharmacol et toxicol.* 1969 27 397–409

Persson, T & B Waldeck: The use of ^{3}H DOPA for studying cerebral catecholamine metabolism. *Acta pharmacol. et toxicol.* 1968, 26 363–372.

Persson, T & B. Waldeck: Is there an interaction between dopamine and noradrenaline containing neurons in the brain? *Acta physiol. scand.* 1970a, 78, 142–144.

Persson, T & B. Waldeck: Further studies on the possible interaction between dopamine and noradrenaline containing neurons in the brain. *European J Pharmacol.* 1970b, in press.

Persson, T & B. Waldeck: Some problems encountered attempting to estimate the catecholamine turnover using labelled tyrosinase. *J Pharm. Pharmacol.* 1970c, in press.

Pomerantz, S. H.. The tyrosine hydroxylase activity of mammalian tyrosine *J Biol. Chem.* 1966, 241 161 168.

Sedvall, G C., V K. Weise & I. J Kopin. The rate of norepinephrine synthesis measured in vivo during short intervals. Influence of adrenergic nerve-impulse activity *J Pharmacol. exp Ther* 1968, 159 274–282.

Sharman, D. F Changes in the metabolism of 3,4-dihydroxyphenylethylamine (dopamine) in the striatum of the mouse induced by drugs. *Brit J Pharmacol.* 1966, 28, 153–163.

Weissmann, A. & B. K. Koe: Behavioural effects of L-α-methyltyrosine, an inhibitor of tyrosine hydroxylase. *Lif Sci.* 1965, 4 1037–1048.

Weissmann, A., B K. Koe & S. Tenen. Antiamphetamine effects following inhibition of tyrosine hydroxylase. *J Pharmacol. Exp Ther* 1966, 151, 339–352.

Acta pharmacol et toxicol. 1970 28 391–405

From the Toxicology Laboratories, AB Astra,
Södertälje, Sweden

The Distribution, Elimination, and Biotransformation of ^{14}C-Cloforex in the Mouse and Rat

By

Åke Ryrfeldt

(Received February 13, 1970)

Abstract: The tissue distribution of radioactivity in mice was studied by whole body autoradiography after oral and intravenous administration of ^{14}C-cloforex. A high uptake and persistence of activity was noted in the lungs and brain. A distinct difference in the distribution of activity in adipose tissue was obtained after oral and intravenous administration. A high activity was found in the fat tissue of mice which had received the drug intravenously but no activity was found following oral administration. This difference may be due to the fact that very little or no cloforex is present in the circulation after oral administration. The excretion of radioactivity was slow. Twenty-four hours after oral administration to rats about 50 % of the administered dose was eliminated, and after 1 week, about 90 %. Most of the activity was eliminated through the kidneys and only small amounts in the faeces and expired air. The tissue retention of activity is certainly of great importance in the slow elimination. The major metabolite in the rat, accounting for about 80 % of the total activity in 8–24 hours urine, was identified as chlorphentermine. The remaining activity was attributed to an unknown metabolite, which seemed to be some conjugate of chlorphentermine. No unchanged cloforex could be found in the urine.

Key-words. Distribution – elimination – biotransformation – appetite depressants.

Cloforex [ethyl-N-(2 p-chlorphenyl-1,1-dimethyl)carbamate] is a drug structurally related to a series of nuclear substituted phenylalkylamines of which amphetamine is the parent compound. Since cloforex has potent appetite depressing properties (LORENZ 1966) it has been widely used in the treatment of obesity.

Investigations by OPITZ & WEISCHER (1966) showed that after oral administration of cloforex to rats chlorphentermine, which lacks the carbethoxy group of the cloforex molecule was excreted in the urine at a slow rate. Chlorphentermine has been shown to be concentrated in certain tissues, e. g. brain and lung, and eliminated at a slow rate (DUBNICK *et al.* 1963 & 19

These studies with cloforex and chlorphentermine indicated that accumulation might occur in the organism after repeated intake. The reason for the slow excretion rate is not known, but in this connection, tissue binding could be of great importance. In order to clarify this, ^{14}C-cloforex was used to study the distribution and elimination of this drug in mice and rats. Some work was also performed to study the biotransformation of cloforex since it influences the tissue uptake and retention of radioactivity

During the course of this investigation it was reported that some compounds used in the treatment of obesity including cloforex, might cause primary pulmonary hypertension in man (GURTNER *et al.* 1968a & b OSTERMAN & TIGNER 1969). It thus became of special interest to investigate whether a high uptake of activity occurred in the lung tissue after administration of cloforex.

Materials and Methods

Radioactive compound.

Cloforex labelled with ^{14}C in the two methyl groups was obtained from the Radiochemical Centre, Amersham, England. The substance had a specific activity of 20 µci/mg (5.20 mci/mmol). The radiochemical purity of the compound was checked by means of thin-layer chromatography as described below The radioactivity of the chromatograms was detected with the aid of a radiochromatogram scanner (Packard, Model 7200) and/or autoradiography Only one radioactive spot was found on the chromatograms.

Autoradiography

Nine male albino mice of the NMRI-strain, weighing 20–25 g, received 1 mg (20 µci) ^{14}C-cloforex in 25 % aqueous Cremophor® EL (Badischa Anilin- & Sodafabrik AB) solution. The solution was given by stomach tube and the mice were killed 5 and 20 minutes, and 1 2, 4, 8,24 48, and 96 hours respectively after administration.

In another series of experiments, five male albino mice of the NMRI-strain, received intravenously into a tail vein 1 mg (20 µci) ^{14}C-clororex in 25 % aqueous Cremophor® EL solution. The animals were killed 5 and 20 minutes and 1 4, and 24 hours respectively after administration of the compound.

The techniques described by ULLBERG (1954), with modifications described below were employed for the studies. The animals were killed with ether anaesthesia and then embedded in a 3 % aqueous solution of carboxymethyl cellulose. The animals were then deep-frozen by immersion for 10 minutes in hexane cooled with solid CO_2. Sagittal sections (40 µ) of the whole body were cut at − 10 and each section was attached to tape (No. 810, *Minnesota Mining and Manufacturing Co.*) and dried at the same temperature for 2–3 days. The tape mounted sections were pressed against X-ray film (Structurix D7 Gevaert) for 3–4 weeks. The films were then developed in Gevaert G 230 X-ray developer for 5 minutes and fixed in Gevaert G-350 for 10 minutes.

Radioactive counting.

The radioactivity was assayed in a Packard Tri-Carb liquid scintillation spectrometer Model 314 EX. Correction for quenching was made by the channels ratio procedure. The counting was performed as to avoid an error larger than ± 3 % (95 % confidence limit) in the total count of each sample.

Scintillation solution I (naphthalene 60 g, PPO 4 g, PPO 4 g, dimethyl-POPOP 0.2 g methanol 100 ml, ethyleneglycol 20 ml, and dioxan ad 1000 ml) was used to assay radioactive samples containing water, scintillation solution II (PPO 4 g, dimethyl-POPOP 50 mg, and toluene ad 1000 ml) to assay organic liquids containing radioactivity and scintillation solution III (phenethylamine 270 ml, methanol 270 ml, PPO 5 g, dimethyl-POPOP and toluene ad 1000 ml) was used to trap and assay radioactive CO_2.

Collection of ^{14}C-activity from rats.

Male Sprague-Dawley rats received ^{14}C-cloforex diluted with carrier (1 + 1) in 25 % aqueous Cremophor® EL solution by stomach tube. The dose of cloforex was 10 mg/kg.

In order to study the excretion of activity in the urine and faeces, the rats were placed in metabolism cages and received water and food *ad libitum*. The faeces were collected during periods of 24 hours for one week and the urine was collected in beakers which were changed after 2, 4 8, 24, 48, 72, 96, 120, 144 and 168 hours respectively During sampling, the beakers were cooled with dry ice and the samples stored at − 10 before the activity was assayed. The faeces were homogenized after the addition of 2.5 ml water per gram and aliquots equivalent to 2.0 g were made alkaline with NaOH to pH 12 and extracted with 15.0 ml benzene. The radioactivity of the organic phase was assayed.

In order to study the excretion of activity in expired air, the rats were placed in glass metabolism cages and received water and food *ad libitum*. Respiratory gases were swept by a stream of air into scintillation solution III (300–400 ml) in scrubber tower The scintillation solution was changed t intervals of 4–6 hours. The activity was determined in accurately measured volumes (10.0 ml) of the solution.

Extraction methods in urine investigations.

The urine (8–24 hrs) was subjected to acid or alkaline extraction. Acid extraction was performed by shaking 2.0 ml of urine diluted with 2 ml water with 15.0 ml benzene after acidification with HCl to pH 1 After centrifugation the organic phase was removed by aspiration and assayed for radioactivity Part of the organic phase was concentrated *in vacuo* and subjected to TLC. Alkaline extraction was performed after being made alkaline with NaOH to pH 12. The urine was also hydrolyzed by acidifying with HCl (pH 0.5–1.0) and refluxing for about 20 hours. The hydrolyzate was subjected to acid and alkaline extraction as described above.

Alkaline extraction f various tissue samples.

Three male rats (Sprague-Dawley) weighing about 300 g, received ^{14}C-cloforex diluted with carrier (1 + 1) in 25 % aqueous Cremophor® EL solution by stomach tube. The dose of cloforex was 10 mg/kg.

Four hours after administration, the rats were killed by decapitation and the

liver and lungs were immediately taken out and homogenized in ice-cold saline with a Potter-Elvehjem homogenizer. The homogenates were made alkaline with 2 M-NaOH (pH 12) and extracted by shaking with benzene for 15 minutes. The organic phases were separated by centrifugation and concentrated *in vacuo* and the concentrates obtained were subjected to TLC.

In vitro study

For incubation studies 9000 × g supernatant of rat liver was used. Male albino Sprague-Dawley rats (200 g) were killed by decapitation, the liver removed as quickly as possible and rinsed in 0.15 M KCl solution. The livers were homogenized in 3 volumes of 0.15 M KCl solution with a Potter-Elvehjem homogenizer and the homogenates were centrifuged at 9000 × g for 20 minutes. The supernatant was used for the incubations.

The substrates, 0.4 µmol ^{14}C-cloforex in solution (the final reaction mixture contained 0.04 % Cremophor® EL in order to dissolve cloforex), was incubated at 30° under continuous shaking in air with 1.0 ml 9000 × g supernatant in 5.0 ml reaction mixture containing 0.04 M phosphate (K) buffer pH 7.4, 0.06 M KCl, 5.0 mM $MgSO_4$, 0.02 M nicotinamide, 5.0 mM glucose-6-phosphate and 0.12 mM NADP (Nicotinamide, glucose-6-phosphate and NADP were supplied by Sigma® Chemical Co.) Aliquots (1.0 ml) were removed from the incubation medium at 0 (15–20 sec.), 5, 30, 60, and 120 minutes respectively after the incubation had started, and put into glass-stoppered centrifuge tubes containing 0.3 ml 2 M NaOH and 0.5 g NaCl. The reaction mixture was extracted by shaking with 15.0 ml benzene and the phases separated by centrifugation. An aliquot of the organic phase (10.0 ml) was removed by aspiration and transferred into another glass-stoppered centrifuge tube which contained 5.0 ml 0.1 M HCl. Extraction was performed as described above and the phases separated by centrifugation. A part of the aqueous phase was removed by aspiration and the radioactivity of this phase was assayed. Concentrates of aliquots of the aqueous phases (4.0 ml) were submitted to TLC and the radioactivity of the chromatograms was detected with the aid of autoradiography.

Thin-layer chromatography (TLC)

TLC was carried out on silica gel plates (DC Fertigplatten Kieselgel F_{254}, E Merck AG) and the plates were developed in sandwich chambers (Chemie-Erzeugnisse und Absorptionstechnik AG, Switzerland). The plates were not activated before use. The following solvent systems were used for developing the chromatograms.

A. n-BtOH : HAc : H_2O (4 : 1 : 5)
B. n-BtOH : NH_3 : H_2O (4 : 1 : 5)
C. EtAc : HAc : H_2O (4 : 1 : 5)
} The organic phases were used.
D. $CHCl_3$

Nitrogen-containing compounds were visualized by pretreatment of the chromatograms with Cl_2-gas and spraying with o-tolidine reagent (o-tolidine 32 mg, KI 200 mg, HAc 6 ml and aq. dest. ad 100 ml). The radioactivity on the chromatograms was detected with the aid of a radiochromatogram scanner (Packard, Model 7200) and/or by pressing X-ray film (Structurix D7 Gevaert) against the silica layer for 4–8 days. The films were developed in Gevaert G-230 X-ray developer for 5 minutes and fixed in Gevaert G-305 for 10 minutes.

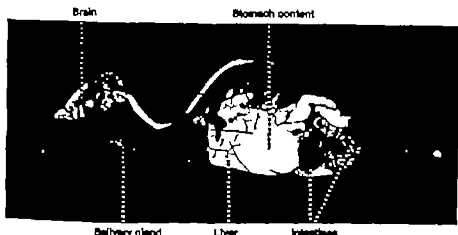


Fig. 1. Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 20 min. after oral administration of ^{14}C-cloforex.

Results

Tissue distribution as revealed by autoradiography in mice.

Five minutes after oral administration of ^{14}C-cloforex, activity could be detected in the liver. Traces of activity were also found in the kidney but most of the radioactivity was localized in the stomach content. After intravenous administration, the highest concentration of activity was found in the liver, kidney, lung, Harder's gland, and particularly in fat tissue. The CNS and myocardium also showed uptake of activity but little activity was found in the blood.

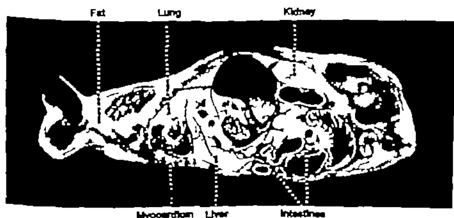


Fig. 2. Autoradiogram showing the distribution of radioactivity (light areas) in mouse 20 min. after intravenous administration of ^{14}C-cloforex.

liver and lungs were immediately taken out and homogenized in ice-cold saline with a Potter-Elvehjem homogenizer. The homogenates were made alkaline with 2 M NaOH (pH 12) and extracted by shaking with benzene for 15 minutes. The organic phases were separated by centrifugation and concentrated *in vacuo* and the concentrates obtained were subjected to TLC.

In vitro study

For incubation studies 9000 × g supernatant of rat liver was used. Male albino Sprague-Dawley rats (200 g) were killed by decapitation, the liver removed as quickly as possible and rinsed in 0.15 M KCl solution. The livers were homogenized in 2 volumes of 0.15 M KCl solution with a Potter-Elvehjem homogenizer and the homogenates were centrifuged at 9000 × g for 20 minutes. The supernatant was used for the incubations.

The substrates, 0.4 µmol ^{14}C-cloforex in solution (the final reaction mixture contained 0.04 % Cremophor® EL in order to dissolve cloforex), was incubated at 30° under continous shaking in air with 1.0 ml 9000 × g supernatant in 5.0 ml reaction mixture containing 0.04 M phosphate (K) buffer pH 7.4, 0.06 M-KCl, 5.0 m M-$MgSO_4$, 0.02 M nicotinamide, 5.0 mM glucose-6-phosphate and 0.12 mM NADP (Nicotinamide, glucose-6-phosphate and NADP were supplied by Sigma® Chemical Co.) Aliquots (1.0 ml) were removed from the incubation medium at 0 (15–20 sec), 5, 30, 60, and 120 minutes respectively after the incubation had started, and put into glass-stoppered centrifuge tubes containing 0.5 ml 2 M-NaOH and 0.5 g NaCl. The reaction mixture was extracted by shaking with 15.0 ml benzene and the phases separated by centrifugation. An aliquot of the organic phase (10.0 ml) was removed by aspiration and transferred into another glass-stoppered centrifuge tube which contained 5.0 ml 0.1 M-HCl. Extraction was performed as described above and the phases separated by centrifugation. A part of the aqueous phase was removed by aspiration and the radioactivity of this phase was assayed. Concentrates of aliquots of the aqueous phases (4.0 ml) were submitted to TLC and the radioactivity of the chromatograms was detected with the aid of autoradiography.

Thin-layer chromatography (TLC)

TLC was carried out on silica gel plates (DC Fertigplatten Kieselgel F254, E Merck AG), and the plates were developed in sandwich chambers (Chemie-Erzeugnisse and Absorptionstechnik AG, Switzerland). The plates were not activated before use. The following solvent systems were used for developing the chromatograms.

A. n-BuOH : HAc : H_2O (4 : 1 : 5)
B. n-BuOH : NH_3 : H_2O (4 : 1 : 5)
C. EtAc : HAc : H_2O (4 : 1 : 5)
} The organic phases were used.
D. $CHCl_3$

Nitrogen-containing compounds were visualized by pretreatment of the chromatograms with Cl_2-gas and spraying with o-tolidine reagent (o-tolidine 32 mg, KI 200 mg, HAc 6 ml and aq. dest. ad 100 ml). The radioactivity on the chromatograms was detected with the aid of a radiochromatogram scanner (Packard, Model 7200) and/or by pressing X-ray film (Structurix D7, Gevaert) against the silica layer for 4–8 days. The films were developed in Gevaert G-230 X-ray developer for 5 minutes and fixed in Gevaert G-305 for 10 minutes.

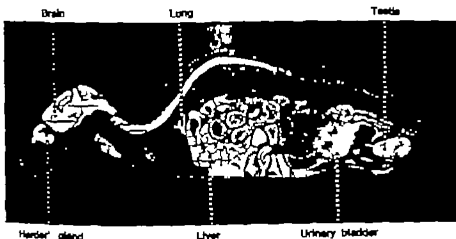


Fig. 5. Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 24 hours after oral administration of ^{14}C-cloforex.

One hour after administration a marked increase of activity could be found in most of the tissues of the mice which had received the drug orally. The highest concentration of activity was found in the CNS, lung, and kidney. Radioactivity was also found in the myocardium. Compared with the mice which had received the drug intravenously the most striking difference was found in the fat. A high activity was found in the fat tissue after intravenous administration but no activity was found following oral administration.

From 2 up to 8 hours after oral administration, the distribution of activity in the various tissues seemed to remain constant, with the highest activity

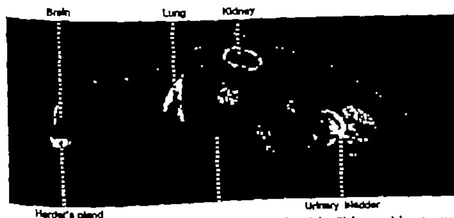


Fig. 6. Autoradiogram showing the distribution of radioactivity (light areas) in mouse 24 hours after intravenous administration of ^{14}C-cloforex.

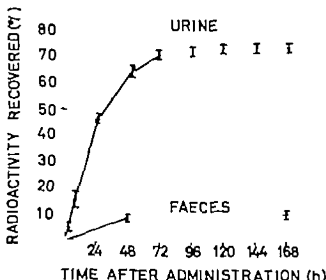


Fig. 7 Mean cumulative amount of radioactivity in the urine and faeces in % of the administered dose of ^{14}C-cloforex, given orally to male Sprague-Dawley rats. The dose of cloforex was 10 mg/kg. All data are means ± S. E. M. (n = 5)

being found in e. g. the lung, kidney and CNS (fig. 3 & 4). After intravenous administration some activity was also found in the fat tissue.

Twenty four hours after oral administration (fig. 5) the activity was seen in e. g. the lung, liver CNS, kidney and especially in the urinary bladder.

Table 1

Amount of radioactivity excreted in expired air after one single oral dose of ^{14}C-cloforex (10 mg/kg body weight), dissolved in Cremophor® EL solution, to male albino rats. Values for samples obtained during periods ending at the indicated times are given as well as the cumulative amounts.

Time (hrs)	Activity excreted (% of dose administered)					
	Rat 1 (257 g)		Rat 2 (173 g)		Rat 3 (200 g)	
		cumul.		cumul.		cumul.
4	0.2	0.2	0.3	0.3	0.1	0.1
8	0.2	0.4	0.3	0.6	0.3	0.4
14	0.5	0.9	0.4	1.0	0.5	0.9
24	0.4	1.3	0.3	1.3	0.6	1.5
31					0.2	1.7
37					0.2	1.9
48					0.1	2.1

After intravenous administration (fig 6) most of the activity had disappeared from the body The urinary bladder however showed a high concentration of activity and a high activity was also observed in the lung. Other tissues showed only traces of activity e. g. CNS, intestinal wall, and testis. Forty eight and 96 hours after administration no activity could be found except for some activity in the urinary bladder

Excretion in urine, faeces and expired air from rats

The excretion of radioactivity in urine faeces and expired air after oral administration of ^{14}C-cloforex is shown in fig. 7 and table 1 The activity appeared rather slowly in the urine. The samples at 2 and 4 hours after administration as a rule contained a low amount. Between 43.3 and 48.5 / of the given dose was excreted in the urine during the first 24 hours. During the 168 hours observation period about 75 / of the administered dose was excreted in the urine.

The amount of radioactivity obtained in the alkaline benzene extracts of the faeces within the 168 hours observation period amounted to about 10 / Most of the activity appeared within the first 48 hours.

The amount of radioactivity excreted in the expired air after oral administration was studied in 3 rats. Two of them were studied for 24 hours and one for 48 hours. The results showed that only small amounts of activity were excreted in the expired air as ^{14}C-labelled CO_2 After 24 hours 1 4 / (mean of 3 rats) of the administered dose of radioactivity was excreted and after 48 hours, 2.1 / (1 rat)

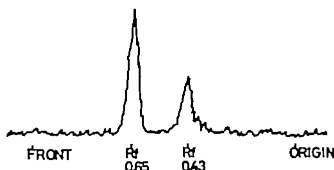


Fig. 8 TLC of urine (0– 4 hrs) collected from a rat treated orally with ^{14}C-cloforex. The plate was developed in n-BuOH HAc H_2O (4 1 5) Radioactivity was located with Packard Radiochromatogram Scanner

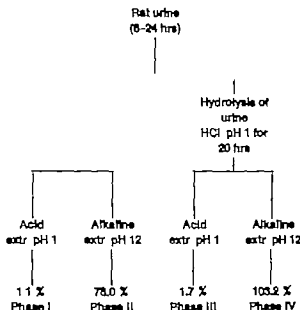


Fig. 9 The values obtained give the radioactivity extracted into the organic phases (benzene) as % of the radioactivity in the urine.

Metabolites in urine from rats

When the urine (8–24 hrs) was subjected to TLC, 2 radioactive peaks could be detected on the chromatograms. The Rf-values of the 2 peaks in the 3 systems were 0 65 and 0 43 (system A) 0 40 and 0 17 (system B), and 0.23 and 0 04 (system C) A radiochromatogram scan of the plate developed in solvent system A is shown in fig. 8. The Rf value of authentic cloforex is 1.00 in all the 3 systems and the Rf-values of chlorphentermine are 0 65 (system A) 0.38 (system B) and 0.26 (system C) None of the radioactive peaks had an Rf value corresponding to cloforex but chlorphentermine could be identified from the Rf values. The results also showed that a metabolite was present in the urine which seemed to be more polar than chlorphentermine

When the urine was extracted with benzene at pH 1 about 1 / of the total radioactivity in the urine sample was extracted into the organic phase and at pH 12 about 80 / was extracted (fig. 9) TLC of the organic phase, obtained after extraction of the urine at pH 1 showed only traces of radioactivity but none of these spots had an Rf value corresponding to unchanged cloforex. One radioactive peak was found by scanning when the organic phase obtained after alkaline extraction was subjected to TLC. This peak could be identified as chlorphentermine from the Rf values in the different systems (table 2).

To determine whether the unknown metabolite could be a conjugate, the urine was hydrolyzed by acid. When a part of the acid hydrolyzate was

Table 2

TLC of the concentrates of the organic phases obtained in the extraction experiments of radioactive rat urine (see fig. 9). The radioactivity was localized by scanning.

	Rf values	
	System A[a])	System D[b])
Cloforex	1.00	0.50
Chlorphentermine	0 65	0 00
Phase I[c])	–	–
Phase II[c])	0.64	0.00
Phase III)	–	–
Phase IV[c])	0.66	0.00

[a]) n-BtOH : HAc H_2O (4 1 5)
[b]) $CHCl_3$
) The phases are designated as in fig. 9

directly extracted with benzene a recovery of about 1–2 / of the total radioactivity was obtained in the organic phase. When another part of the hydrolysate was made alkaline (pH 12) and extracted with benzene, a recovery of about 100 / was obtained (fig. 9) TLC of this phase showed that only chlorphentermine was present (table 2) This showed that the unknown metabolite could be hydrolyzed to chlorphentermine.

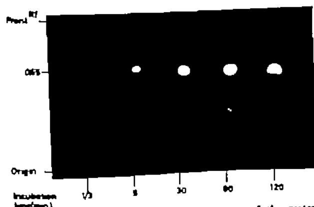


Fig. 10. Autoradiogram of thin-layer chromatogram of the water phases obtained in the extraction procedures in the incubation studies of ⁴C-cloforex with 9000 × g rat liver supernatant. The autoradiogram shows that the amount of chlorphentermine formed (Rf 0 65) increases with increasing incubation time. Solvent system. A [n-BtOH HAc H_2O (4 1 5)]

Investigation of metabolites in various tissue samples from rats

TLC, in system A, of the organic phases obtained after alkaline extraction of the tissue homogenates of the brain, liver and lung indicated that no unchanged cloforex was present in these tissues 4 hours after oral administration of cloforex. Only radioactivity attributed to chlorphentermine could be detected on the plates both by scanning and autoradiography

Incubation study with rat liver 9000 × g supernatant

Incubation of ^{14}C-cloforex with 9000 × g rat liver supernatant showed that cloforex was easily degraded to chlorphentermine. The results from an incubation study are shown in fig. 10 and table 3 In order to ensure that no metabolite was formed which was not extracted into the organic phase after making the incubation medium alkaline, aliquots of the incubation medium were applied directly to a TLC plate after 0 15 30 60 120, and 180 minutes incubation. Only 2 radioactive peaks could be detected by scanning. One peak corresponding to authentic cloforex decreased in size with increasing incubation time, while the other peak, increased in size with time and corresponded to authentic chlorphentermine

Table 3

Incubation of ^{14}C-cloforex with 9000 × g rat liver supernatant. After different time intervals, aliquots of the incubation medium were extracted with benzene at pH 12. The organic phase was then extracted with 0.1 M HCl In the table, the amount of radioactivity excreted into the acidic aqueous phase is given as % of the total amount of radioactivity in the incubation medium. This radioactivity is a direct measure of the chlorphentermine formed.

Incubation time (min.)	Amount of radioactivity excreted into aqueous phase (%)
0 (20 sec.)	0.9
5	9.5
30	50.8
60	71.8
120	76.5

Discussion

The tissue distribution of radioactivity as revealed by autoradiography after administration of cloforex showed high uptake of activity in e g the lung, CNS, and liver both after oral and intravenous administration. The carbethoxy group in the cloforex molecule is very resistant to acid and alkaline hydrolysis but in the body this group proved to be unstable and cloforex was easily degraded to chlorphentermine. Studies *in vitro* showed that 9000 × g rat liver supernatant was active in degrading cloforex to chlorphentermine. Preliminary investigations indicated that only chlorphentermine and no cloforex was present in tissue extracts of the lung, brain, and liver taken from rats 4 hours after oral administration of cloforex. The present studies therefore indicate that most of the activity found in various tissues in the autoradiographic studies may be attributed to chlorphentermine.

The high activity found in the fat after intravenous administration can be attributed to unchanged cloforex, since a massive dose is injected into the systemic circulation during a short time interval and since cloforex is more lipid soluble than chlorphentermine. It is not very likely that the activity found in the fat is attributable to chlorphentermine since DUBNICK *et al* (1968) showed that only very small amounts of activity were found in fat tissue after intravenous administration of ^{14}C-chlorphentermine into mice. Lipid soluble metabolites of cloforex may be responsible for this activity in the fat, but so far no evidence has been found for the existence of such metabolites in appreciable amounts.

As it has been reported that some anorexigenic drugs may cause primary pulmonary hypertension, it seemed of interest to determine whether there was a high uptake and retention of activity in lung tissue after administration of cloforex. Conclusions concerning the distribution of radioactivity at cellular or subcellular level cannot be drawn from the present experiments. This may be possible with microautoradiography and such a study is planned. The uptake of several amines in the lungs has been noted e. g. propranolol, a β-receptor blocking agent (MASUOKA & HANSSON 1967) and tricyclic antidepressants such as amitriptyline and imipramine (HANSSON & CASSANO 1966). Para chloramphetamine reaches a lung concentration about 5 times that of amphetamine (FULLER & HINES 1967). The uptake of amines in the lungs is certainly influenced by the lower pH in the pulmonary extravascular space as compared with the pulmonary vascular pH (EFFROS & CHINARD 1969). It seems, however that certain amines e. g. derivatives of p-chlorophenylethylamine show a greater retention in the lungs than can be explained solely by a pure pH-effect. The significance of this retention in the aetiology of primary pulmonary hypertension is not known.

The investigations by OPITZ & WEISCHER (1966) indicated that chlor

phentermine was excreted at a slow rate after administration of cloforex. They found that after one oral dose of cloforex to rats, about 40 % of the administered dose was found in the urine as chlorphentermine in the course of 6 days. Only the excretion in the urine was studied. The slow excretion was confirmed by my own investigations with cloforex, in which studies in rats showed that about 90 % of the administered dose of activity was excreted within 1 week, the main excretion occurring during the first 2 days.

After administration of ^{14}C-cloforex the major metabolite in the urine was shown to be chlorphentermine which has proved to be very stable to enzymatic degradation (DUBNICK *et al.* 1963). The chlorine in the para-position blocks the aromatic hydroxylation, and oxidative deamination is hindered by the 2-methyl groups in the aliphatic chain. It was soon found in my own investigations with ^{14}C-cloforex that the chlorphentermine formed was further transformed in appreciable amounts. In the urine, a metabolite was found which was more polar than chlorphentermine, not extractable into benzene and hydrolyzed by acid to chlorphentermine. This indicated that the unknown metabolite might be a conjugate between some endogenous substance and the amino group in the chlorphentermine molecule. DUBNICK *et al.* (1968) have also stated that chlorphentermine probably undergoes some conjugation reaction. Possible metabolic pathways were N-acetylation or the formation of an N-glucosiduronide of chlorphentermine. Their results indicated, however, that the metabolite found could hardly be an N-glucosiduronide of chlorphentermine or acetylated chlorphentermine.

REFERENCES

Dubnick, B., O. A. Leeson, R. Leverett, D. F. Morgan & O. E. Phillips: Sympathomimetic properties of chlorphentermine: metabolism, metabolic effects, interaction with reserpine and biogenic amines. *J. Pharmacol. exp. Ther.* 1963, 140, 85–92.

Dubnick, B., C. A. Towne, I. M. Hartigan & O. E. Phillips: Distribution and metabolism of chlorphentermine-^{14}C in rats and mice. *Biochem. Pharmacol.* 1968, 17, 1243–1250.

Effros, R. M. & F. P. Chinard: The in vivo pH of the extravascular space of the lung. *J. Clin. Invest.* 1969, 48, 1983–1996.

Fuller, R. M. & C. W. Hines: Tissue levels of chloramphetamine in rats and mice. *J. Pharm. Sci.* 1967, 56, 302–303.

Gurtner, H. P., M. Gertsch, C. Salzmann, M. Scherrer, P. Stucki & F. Wyss: Häufen sich die primär vasculären Formen des chronischen Cor pulmonale? I. *Schweiz. Med. Wschr.* 1968a, 98, 1579–1589.

Gurtner, H. P., M. Gertsch, C. Salzmann, M. Scherrer, P. Stucki & F. Wyss: Häufen sich die primär vasculären Formen des chronischen Cor pulmonale? II. *Schweiz. Med. Wschr.* 1968b, 98, 1695–1707.

Hansson, E. & G. B. Cassano: Distribution and metabolism of antidepressant drugs. *Proceedings of the first international symposium on antidepressant drugs*. Excerpta Medica International Congress Series No. 122, Milan, April 1966, pp. 10–22.

Lorenz, D.: Pharmakologie moderner Appetitzügler. *Arch. Pharm.* (Weinheim), Mitteilungen 1966, 12, 269–282.

Masuoka, D. & E. Hansson. Autoradiographic distribution studies of adrenergic blocking agents. II. ^{14}C-propranolol, a β-receptor-type blocker. *Acta pharmacol. et toxicol.* 1967, 25, 447–455.

Opitz, K. & M. L. Weischer: Die Ausscheidung anorexigener Phenylalkylamine mit dem Harn. *Arzneimittelforschung* 1966, 16, 1311–1313.

Osterman, K. & K.-B. Tegner: Fall av pulmonell hypertension. *Läkartidningen* 1969, 66, 5194–5195.

Ullberg, S.: Studies on the distribution and fate of ^{35}S-labelled benzylpenicillin in the body. *Acta Radiol.* Suppl. 118, 1954.

Acta pharmacol et toxicol 1970 **28**, 406–412

From the Department of Pharmacology University of Göteborg,
Göteborg, Sweden

Failure to Demonstrate Monoamine Oxidase Inhibition by Glyceryl Trinitrate in Vivo

By

Bertil Waldeck

(Received February 27 1970)

Abstract Glyceryl trinitrate (GTN) or the monoamine oxidase (MAO) inhibitor nialamide was given to rats or mice which then received ^{14}C-tyramine. Nialamide but not GTN increased the yield of the ^{14}C-octopamine formed in the heart. In nialamid pretreated mice GTN possibly caused a slight increase in ^{14}C-octopamine. GTN given before or after ^{3}H-noradrenaline had no effect on the uptake and retention of this amine in the mouse heart. Neither did it affect the uptake of ^{3}H-metaraminol in this organ. It is concluded that GTN has no effect on the MAO responsibl for the *in vivo* metabolism of tyramine and noradrenaline. The existence of MAO systems with different substrate and inhibitor sensitivity is discussed.

Key-words: Glyceryl trinitrate – monoamine oxidase.

There are conflicting reports regarding the effects of glyceryl trinitrate (GTN) on the metabolism of noradrenaline (NA) Using a manometric method, OGAWA *et al.* (1967) demonstrated monoamine oxidase (MAO) inhibiting properties of GTN *in vitro* as well as *in vivo* MAO inhibition by GTN *in vitro* was confirmed by KALIN & KYLIN (1969) Using a histochemical method, however no signs of MAO inhibition nor any indication of interference by GTN with the uptake, storage and metabolism of NA in the adrenergic neuron could be observed *in vivo* (KALIN *et al* 1969).

Tyramine (TA) which is a good substrate for MAO (BLASCHKO *et al* 1937) is converted to octopamine (OA) within the adrenergic neuron (CARLSSON & WALDECK 1963b FISCHER *et al.* 1964). After inhibition of the MAO the yield of OA from TA in the mouse heart increases about fourfold (CARLSSON & WALDECK 1963b). In the present study the effect of GTN on the formation of ^{14}C-OA from ^{14}C TA has been investigated and compared with that of the MAO inhibitor nialamide. Furthermore, the possible interference by

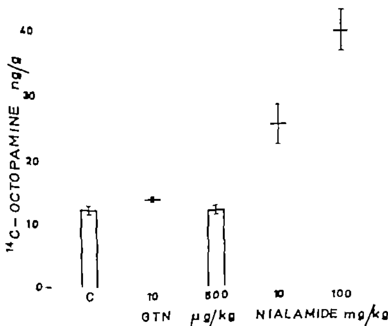


Fig. 1. Effect of glyceryl trinitrate (GTN) and of the MAO inhibitor nialamide on the formation of ^{14}C-octopamine from ^{14}C-tyramine in the rat heart. GTN, 10 or 500 µg/kg was given intraperitoneally 15 min. before or nialamide 10 mg/kg intraperitoneally 30 min. before or 100 mg/kg intraperitoneally 2 hrs before the intravenous administration of 10 µg/kg ^{14}C-tyramine. Rats which received only ^{14}C-tyramine served as controls (C). Thirty minutes after the administration of the labelled amine the animals were killed and ^{14}C-octopamine in the heart determined. The mean ± S.E.M. of 4–5 rats are shown.

GTN with the uptake and storage mechanisms of the adrenergic neuron has been investigated using labelled NA and metaraminol.

Methods

Male Sprague Dawley rats weighing about 200 g or female white mice with an average weight of 20 g were used. Tissue extraction in 0.4 N perchloric acid and subsequent separation of the labelled amines on cation exchange columns were made as described by CARLSSON & WALDECK (1963a & 1965). The acidic eluates were prepared for liquid scintillation counting by freeze drying (WALDECK 1968). In the experiments with ^{3}H NA, however 4 ml of the eluates were added to 10 ml of special scintillation solvent mixture Insta-Gel® supplied by Packard Instrument Company. The following drugs were used. Glyceryl trinitrate in a 1 % alcoholic solution (in the high dose range, however 10 % solution was used in order to avoid alcohol intoxication), nialamide, tyramine-2-^{14}C (as hydrochloride, 50 mci/mmole or as the hydrobromide, 5 mci/mmole), DL-noradrenaline-7-^{3}H 6.9 ci/mmole, and DL-metaraminol-7-^{3}H (metaraminol = metaradrinum NFN), 4.6 ci/mmole. All drugs were dissolved in saline.

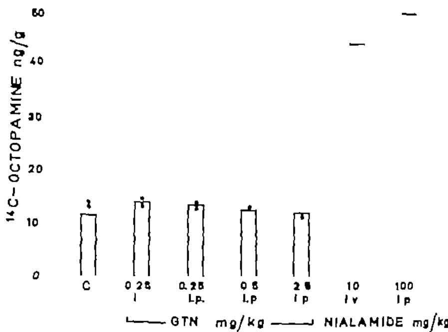


Fig. 2. Effect of glyceryl trinitrate (GTN) and of the MAO inhibitor nialamide on the formation of ^{14}C-octopamine from ^{14}C-tyramine in the mouse heart. GTN in various doses was given intraperitoneally (in one case intravenously) 15 min. before or nialamide 10 mg/kg intravenously 15 min. before or 100 mg/kg intraperitoneally 2 hrs before the intravenous administration of 20 µg/kg ^{14}C-tyramine. Mice which received only ^{14}C-tyramine served as controls (C). Thirty minutes after the administration of labelled amine the animals were killed and ^{14}C-octopamine in the heart determined. Each point represents the pooled hearts from six mice.

Results

Rats were given GTN 10 or 500 µg/kg intraperitoneally 15 min. before or nialamide 10 mg/kg intraperitoneally 30 min. before or 100 mg/kg intraperitoneally 2 hrs before the intravenous administration of 10 µg/kg ^{14}C TA. After another 30 min. the animals were killed and their hearts removed and analyzed for ^{14}C-OA. Rats which received only ^{14}C TA served as controls. GTN did not significantly change the yield of ^{14}C-OA (fig. 1). In contrast, nialamide 10 and 100 mg/kg increased the yield of ^{14}C-OA by 2 and 3 times respectively ($P < 0.001$)

Similarly mice were pretreated with GTN or nialamide in various doses before ^{14}C TA. In this experiment, however the low dose of nialamide was given intravenously and 15 min. before ^{14}C TA. One group received *GTN* intravenously The dose of ^{14}C TA was 20 mg/kg (fig. 2) In this series of

Table 1

Effect of glyceryl trinitrate (GTN) on the formation of ^{14}C-octopamine from ^{14}C-tyramine in the heart of nialamide pretreated mice. Nialamide, 100 mg/kg, was given intraperitoneally 2 hrs before and GTN in various doses 15 min. before the intravenous injection of 20 µg/kg ^{14}C-tyramine. Thirty minutes after the labelled amine had been given the animals were killed and ^{14}C-octopamine in the heart determined. The mean of 5 experimental groups each comprising 6 animals are shown.

	GTN mg/kg		
	none	0.25	2.5
^{14}C-octopamine ng/g	29.9	34.8	40.2
S.E.M.	± 0.9	± 0.9	± 4.6

experiment also GTN failed to change the yield of ^{14}C-OA from ^{14}C-TA, while ^{14}C-OA in the nialamide pretreated animals increased by about four times.

In the the next experiment nialamide 100 mg/kg was given intraperitoneally to mice 2 hrs before the intravenous administration of ^{14}C TA, 20 µg/kg. In addition some animals received GTN 0.25 or 2.5 mg/kg intraperitoneally 15 min. before ^{14}C TA. After another 30 min. the animals were killed and ^{14}C-OA in the heart determined. If anything, GTN slightly increased the yield of ^{14}C-OA from ^{14}C TA in nialamide pretreated mice (table 1)

In another experiment mice received GTN in various doses intraperitoneally either 15 min. before or 15 min after the intravenous administration of ^{3}H-NA, 1 µg/kg. The animals were killed 30 and 60 min. respectively after

Table 2

Effect of glyceryl trinitrate (GTN) on the uptake and retention of ^{3}H-noradrenaline in the mouse heart. Various doses of GTN were given intraperitoneally either 15 min. before or 15 min. after the intravenous administration of 1 µg/kg ^{3}H-noradrenaline. The animals were killed 30 and 60 min., respectively after the labelled amine was given and the heart content of ^{3}H-noradrenaline was determined. The values are the mean of 5 experimental groups each comprising 6 animals.

	GTN mg/kg					
	before			after		
	none	0.25	2.5	none	0.25	2.5
^{3}H-noradrenaline ng/g	3.12	2.55	2.83	2.53	2.40	
S E.M.	± 0.56	± 0.19	± 0.32	± 0.45	± 0.[illegible]1	

Table 3

Effect of glyceryl trinitrate (GTN) on the uptake of ^{3}H-metaraminol in the mouse heart. Various doses of GTN were given intraperitoneally 15 min. before the intravenous administration of 0.1 μg/kg ^{3}H-metaraminol. Thirty minutes after the labelled amine was given the animals were killed and the heart content of ^{3}H-metaraminol determined. The values are the mean of 4 experimental groups each comprising 6 animals.

	GTN mg/kg			
	none	0.25	2.5	25
^{3}H-metaraminol ng/g	0.24	0.24	0.27	0.24
S.E.M.	± 0.016	± 0.022	± 0.016	± 0.013

administration of the labelled amine. ^{3}H-NA in the hearts was determined. Animals receiving ^{3}H NA alone served as controls. GTN had no significant effect on the amount of ^{3}H-NA recovered from the heart (table 2).

Finally GTN in various doses was given intraperitoneally to mice 15 min. before the intravenous administration of ^{3}H-metaraminol, 0 1 μg/kg. The animals were killed 30 min. later and the ^{3}H-metaraminol in heart was determined. No effect of GTN on the uptake and retention of ^{3}H-metaraminol could be detected (table 3).

Discussion

In contrast to an established MAO inhibitor namely nialamide, GTN in the doses used did not increase the yield of ^{14}C-OA formed from ^{14}C TA in the heart. It should be noted that the dose range in the present investigation covers not only that used by OGAWA *et al.* (1967) but also includes doses which are 100 times higher

This lack of effect of GTN in the present experiments, however could be due to effects by GTN opposing the formation and/or accumulation of ^{14}C-OA such as inhibition of dopamine β-hydroxylase, inhibition of the uptake and storage of amines in the adrenergic neuron and haemodynamic changes. Inhibition of the β-hydroxylation should be revealed if the MAO had been blocked by a specific inhibitor before the administration of GTN. In the experiment with nialamide given before GTN no reduction in the yield of ^{14}C-OA could be observed as compared with animals which had received nialamide only In fact the ^{14}C-OA tended to be higher in animals which received GTN in addition to nialamide. No explanation for this phenomenon can be offered at present. However it excludes the possibility that the haemodynamic changes caused by GTN would oppose the effect of a

possible MAO inhibition by this drug. GTN when given either before or after ^{3}H-NA did not influence the uptake or retention of this amine. This is in agreement with the histochemical observations of KALIN *et al* (1969) Nor was it possible to influence the uptake of the metabolically more stable ^{3}H-metaraminol, even with doses up to 25 mg/kg of GTN

It will thus appear that there are no indications of an MAO inhibition of the *in vivo* metabolism of NA and TA by GTN in the heart of the rat and mouse (cf KALIN *et al* 1969) Recently however two MAO systems with different substrate and inhibitor sensitivity have been demonstrated (JOHNSTON 1968 HALL *et al.* 1969) An interesting interpretation of the results obtained by OGAWA *et al.* (1967) would then be that GTN inhibits a fraction of MAO which is of less importance for the metabolism of NA and TA in the living tissue. The fact that these authors were unable to obtain an inhibition of more than 60 per cent arbues in favour of this view

Acknowledgements

This research was supported by the Swedish Medical Research Council (grant No B70-14X 155-06B) I am indebted to Mrs. Barbro Alding and Mrs. Lena Löfberg for the skilful technical assistance.

REFERENCES

Blaschko, H., D Richter & H. Schlossmann: The oxidation of adrenaline and other amines. *Biochem. J* 1937 31 2187–2196.

Carlsson, A. & B. Waldeck: On th role of the liver catechol-0-methyl transferase in the metabolism of circulating catecholamines. *Acta pharmacol. et toxicol* 1963a, 20 47–55.

Carlsson, A. & B. Waldeck. β-hydroxyl tion of tyramine in vivo. *Acta pharmacol. et toxicol* 1963b, 20 371–374

Carlsson, A. & B. Waldeck: Mechanism of mine transport in the cell membranes of the adrenergic nerves. *Acta pharmacol et toxicol.* 1965, 22, 293–300.

Fischer J E., J Musacchio, I. J Kopin & J Axelrod. Effects of denervation on the uptake and β-hydroxylation of tyramine in th rat salivary gland. *Lif Sci.* 1964, 3 413–419

Hall, D. W R., B. W Logan & G H Parsons. Further studies on the inhibition of monoamine oxidase by M & B 9302 (Chlorgyline)-I substrate specificity in various mammalian species. *Biochem. Pharmacol.* 1969 18, 1447–1454.

Johnston, J P Some observations upon a new inhibitor of monoamine oxidase in brain tissue. *Biochem. Pharmacol.* 1968, 17 1285 1297

Kalin, M & B. Kylin Organic nitrate explosives as monoamine-oxidase inhibitors. *Arch. Environm. Health* 1969 18 311 314.

Kalin, M., B. Kylin & T. Malmfors: The effect of nitrate explosives on nerves. *Arch. Environm. Health* 1969, 19, 32–35.

Ogawa, K., S. Gudbjarnason & R. J. Bing: Nitroglycerin (glyceryl trinitrate) as a monoamine oxidase inhibitor. *J. Pharmacol. exp. Ther.* 1967, 155, 449–455.

Waldeck, B.: Preparation of aqueous samples for liquid scintillation counting with the aid of freeze-drying. *Acta physiol. scand.* 1968, 79, 9–10A.

Acta pharmacol. et toxicol. 1970 **28**, 413–424

From the Pharmacological Laboratory Alfred Benzon Ltd., Copenhagen, Denmark

Percutaneous Absorption of Boric Acid from Boron-Containing Preparations in Rats

By

Gyda H. Nielsen

(Received October 27 1969)

Abstract: An aqueous jelly and two oleaginous ointments containing less than 3 % boric acid w/v were applied on the skin of anaesthetized rats. When application was made on the *intact* skin of the dorsal surface (about 28 cm²) determination of boric acid concentrations in the urine 8 hours later showed no or slight increases above the normal level. When the skin was *damaged* the ointments caused increased excretion up to 4–8 times that of the excretion found in control animals, while the jelly caused 34 times increase. When an enclosed skin area of 4.3 cm² was damaged and treated, and simultaneous recording of boric acid recovery in both the urine and applied preparations (jelly and one ointment) was made 5 hours later about 98 % boric acid still remained in the ointment, but only 25 % in the jelly. The recoveries in the urine were 1 % and 25 % respectively indicating slight absorption and rapid excretion in the case of the oleaginous ointment, and high absorption with inadequate excretion when the aqueous jelly was applied.

Key-words: Boric acids – ointments – skin absorption – rats.

When boric acid is absorbed by the organism most of the amount absorbed is rapidly excreted through the kidneys (KENT & MCCANCE 1941 PFEIFFER *et al.* 1945 DRAIZE & KELLEY 1959).

Thus, by the administration of boric acid the extent to which absorption takes place may usually be seen from the rise in the urinary boric acid concentration. While this has been known for many years, some disagreement still prevails concerning the capacity of boric acid to penetrate through intact skin. This is partly due to the fact that the methods used for measuring boric acid in the urine have often lacked the sensitivity necessary for disclosing minor rises above the normal level (FISHER *et al.* 1955)

On the other hand, the view that through injured skin boric acid can into the organism – to a smaller or greater extent, depending

the injury – seems to be generally accepted, while the influence of the vehicle on the degree of absorption is less well known.

The objects of the present study were: 1) To investigate whether boric acid is capable of penetrating through the intact skin of the rat, and 2) to establish the dependence on the nature of the vehicle of boric acid absorption through severely damaged skin.

Methods

Experimental animals

Female Wistar rats weighing from 160 to 200 g (procedure A) or 170 to 225 g (procedure B) were used. Care was taken that the mean body weight of corresponding control and treatment groups did not differ significantly.

Since with conscious rats, several sources of error had been revealed, the animals were anaesthetized. It was found that pentobarbital anaesthesia did not significantly affect the amount of urine excreted during the experimental period.

Preparations.

I. Control preparation consisting of 10 % methylcellulose (celacol®) in water (pH approximately 7.4).

II. An aqueous boric acid jelly containing about 2.5 % boric acid w/v in the above medium (pH approximately 5.8, viscosity at 28° 76000 cp, at 31° 90000 cp).

III. Control preparation consisting of a mixture of animal and mineral fats in the ratio of 1 : 4 (pH approximately 6.3).

IV. An oleaginous boric acid ointment containing a boric acid-borate buffer corresponding to about 2.8 % boric acid w/v in medium III (pH approximately 6.6; viscosity at 28° 40000 cp, at 31° 28000 cp). This ointment was tested according to procedure A only (see below).

V. Natusan® ointment (commercially available), differing from preparation no. IV solely by linkage of the boric acid to polyvalent alcohols. The amount of these constitutes 12 per cent of the total weight. (pH approximately 5.0; viscosity at 28° 48000 cp, at 31° 32000 cp).

Procedure A. Recovery of boric acid from the urine only

A standard volume of 10 ml of tap water per rat was given orally.

About 10 minutes later the rat was anaesthetized by intraperitoneal injection of pentobarbital (mebumalum NFN) sodium, 35 mg/kg. If required, supplementary doses were given later intramuscularly or intraperitoneally. Using a clipper the hairs along the entire dorsal surface (about 28 cm²) were carefully removed without damaging the skin.

In the experiments with *intact* skin 2.0 ml of the preparation was then applied from a syringe. The preparation was evenly distributed over the depilated area (layer thickness approximately 0.7 mm).

Immediately afterwards the rat was placed on its stomach in a wire cage provided with a urine-collecting device. Urine was collected five and eight hours after the application. At the end of this period the rat was sacrificed.

In the experiments with severely *damaged* skin the damage was produced by energetic brushing of the depilated skin with a wire brush. When this is always done by the same person, lesions of the same severity may be obtained in all the animals. The further treatment of these rats was the same as described for those with intact skin.

Procedure B Recovery of boric acid from both urine and preparation

On the day before the experiment the rat was anaesthetized with ether the region behind the shoulders was depilated, and an oval plastic ring, enclosing an area of about 4.3 cm² was glued on to the depilated skin.

On the day of the experiment water loading was performed as usual. The rat was anaesthetized with pentobarbital, and 1.0 ml of the preparation was distributed from weighed syringe over the area inside the ring (layer thickness approximately 2.3 mm). The exact weight of the preparation applied was recorded.

Five hours later the urine was collected, and the preparation within the ring was transferred quantitatively to a titration vessel by means of a silicon-dioxide (aerosil®) jelly in tetrachloromethane.

Quantitative determinations of boron in urine and preparations.

The urine samples were heated with concentrated sulphuric acid and dianthrimide, which is a specific and highly sensitive reagent for the determination of boron. The absorbance difference between the sample and a reagent blank was read in spec trophotometer at wave-length 620 mμ. The method was elaborated especially for use in blood and urine and allows of a determination of values as low as 1 μg boron per millilitre, with an analytical accuracy of 1–1.5 per cent (HOLMEN-CHRISTENSEN, personal communication).

Because of the fat contained in the preparations the above method could not be used directly for the determination of their boric acid content. For that reason electrometric titration in pyridine in the presence of glycerine was used. The determination was performed by automatic titration (Titrigraph, Radiometer). In this way about 0.2 mg H_3BO_3 can be determined, when 0.1 N-NaOH (1 ml ∼ 0.006184 g H_3BO_3) is used. This means that the boric acid content in 1 ml preparation (25–28 mg) was determined with an accuracy of about 0.7–0.8 %. Titration of the rather strong glycerine-boric acid ensured the specificity of the method, since no other substances with similar properties were present in the titration mixtures.

With regard to the quantitative transfer of preparations from the skin of the rats, preliminary experiments, in which the preparations applied were immediately transferred to the titration vessel and titrated directly without further treatment, had shown that this could be done without any demonstrable loss of boric acid and that the aerosil jelly did not interfere with the analytic results. Reproducibility was satisfactory as is evident from the low standard errors shown in tabl 3.

Results

Table 1 shows the mean values (± S. E. M.) of all groups regarding urine volume and boron concentration in the urine, to the left the results from rats with intact skin are shown and to the right, those from rats with damaged skin.

In the upper part which comprises the aqueous preparations, mean concentrations of boron in the urine from "placebo"-treated rats are seen to vary from 4 to 13 μg/ml. When the skin is intact the corresponding values from rats treated with boric acid jelly lie between 7 and 13 μg/ml. When the sk *is severely damaged, however this level is raised to 170–390 μg/ml.*

As regards the ointments (lower part of the table), mean control

Table 1

All rats: *Urine volume* and boron concentration in the urine. Mean values ± S. E. M. Treatment A. Preparation. 2 *ml*. Skin area. approximately 28 cm² *Time*: 8 hours. Treatment B Preparation. 1 ml. Skin area. 4.3 cm² Time: 5 hours.

Preparation	Excreted	Intact skin A 0–5 hours	Intact skin A 5–8 hours	Intact skin B 0–5 hours	Severely damaged skin A 0–5 hours	Severely damaged skin A 5–8 hours	Severely damaged skin B 0–5 hours
I. Aqueous base (control)		N = 9		N = 5	N = 7		N = 10
	urine ml	6.5 ± 1.0	4.3 ± 0.6	6.7 ± 0.9	3.8 ± 0.7	3 1 ± 0.6	6.6 ± 0.3
	boron conc. μg/ml	6.0 ± 2.2	9 4 ± 2.7	4.2 ± 1 4	9.6 ± 2.3	13.3 ± 2.0	3.9 ± 0.7
II. Boric acid jelly		N = 8		N = 5	N = 15		N = 15
	urine ml	5.1 ± 1.1	4 7 ± 0.8	5.5 ± 0.6	4.0 ± 0 7	3.4 ± 0.5	6.7 ± 0.4
	boron conc. μg/ml	12.8 ± 2.5 ($P = 0.05$)	11.6 ± 2.0	7.2 ± 1.2	386 ± 51 ($P \ll 0.001$)	454 ± 37 ($P \ll 0.001$)	173 ± 26 ($P < 0.001$)
III. Oleaginous base (control)		N = 6		N = 5	N = 8		N = 10
	urine ml	7.0 ± 1.7	2.4 ± 0.5	6.7 ± 1.2	8.7 ± 0.6	2.4 ± 0.3	5.3 ± 0.3
	boron conc. μg/ml	8.0 ± 1.0	11.2 ± 1.5	2.6 ± 0.5	6.4 ± 1.0	10.4 ± 2.3	5.1 ± 0.7
IV Boric acid/borate ol. ointment		N = 6			N = 8		
	urine ml	3.8 ± 1.0	4.5 ± 1.3		7.8 ± 1.0	2.2 ± 0.4	
	boron conc. μg/ml	10 6 ± 3.1	11.2 ± 3.4		14.1 ± 1.9 ($P < 0.01$)	45 ± 11 ($P < 0.01$)	
V Complex Boric acid/borate ol. ointment		N = 9		N = 5	N = 15		N = 12
	urine ml	6.4 ± 1.4	3.9 ± 0 8	5 4 ± 0.3	6 9 ± 0 6	3 6 ± 0.7	7.1 ± 0.4
	boron conc. μg/ml	8.0 ± 2.3	9.3 ± 1 4	4.0 ± 0.7	36 ± 12 ($P = 0.02$)	89 ± 22 ($P < 0.001$)	11 0 ± 2.4 ($P < 0.05$)

boron concentrations between 3 and 11 µg/ml were found. About the same figures were obtained when treatment with boric acid-ointments was carried out on intact skin (4–11 µg/ml), while treatment on damaged skin caused a significant rise in concentration, both when the larger skin area, 28 cm² was involved (IV 14–45 µg/ml $P < 0.01$ V 36–89 µg/ml, $P < 0.01$–0.02), and when the smaller area of 4.3 cm² was treated. (V 11 µg/ml, $P < 0.05$).

The total boric acid excretion in the experimental period was calculated from the figures in table 1 and related to the body weight of the rats, the mean results with corresponding standard errors are shown in fig. 1 The figure demonstrates that the excretion of boric acid is low and the variations are small in the groups with intact skin, regardless of their treatment.

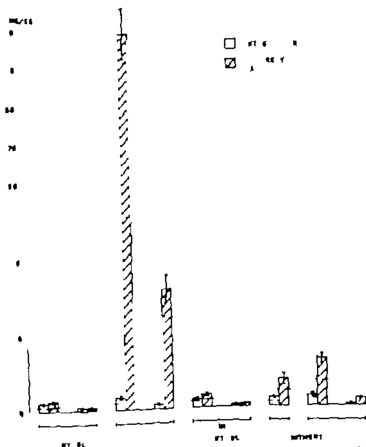


Fig. 1. All rats: Total excretion of boric acid (mg/kg body weight) calculated from urinary boron concentration. Mean values ± S.E.M.
Treatment A. Preparation: 2 ml. Skin area, approximately 28 cm². Urine collected at 5 (dotted lines on columns) and 8 hours.
Treatment B Preparation. 1 ml. Skin area. 4.3 cm². Urine collected at 5 hours.

When the skin is damaged, all of the boric acid-containing preparations caused a significant increase in boric acid excretion with treatment A. However the difference between the increases caused, on the one hand, by the aqueous jelly and on the other hand by the ointments, is considerable and statistically highly significant ($P \ll 0.001$) The increases brought about by each of the two ointments differ less, but are still significant ($P < 0.05$).

The proportional heights of the B-columns, though lower as a whole, are very much like those of the A-columns.

In tables 2 and 3 (treatment A and B respectively) the amounts of boric acid recovered are compared with the amounts applied, but only for the rats with severely damaged skin.

In table 2 the difference between the preparations becomes even more outstanding: the dose of boric acid applied in the jelly was *12 % lower* yet the excretion was much higher than in the ointment-treated groups, so that one third of the dose was recovered in the urine within 8 hours, while the corresponding figures were approximately 1½ % for ointment IV and 3 % for ointment V

With treatment B (table 3) about one quarter of the boric acid from the jelly was recovered in the urine and another quarter in the preparation, which means a total recovery of only about 50 %

The total recovery from ointment V was approximately 99 % of which only about 1 % was found in the urine.

Fig. 2 gives all the recoveries of boric acid related to the amounts applied, and for the sake of comparison, the rats with intact skin are included.

The lower part of the figure (treatment B) shows that by far the largest part of the boric acid is still found in the preparation at the end of the experiment, except when the aqueous jelly is applied on damaged skin, in which case about one half of the boric acid is not found either in the urine or in the remaining jelly

Discussion

The results of the present study show in agreement with those of some previous investigations (DRAIZE & KELLEY 1959 PFEIFFER *et al.* 1945) that boric acid is capable of penetrating the undamaged skin only to a small extent, and that the nature of the vehicle in which the substance is applied is here of little importance, at least within the pH range chosen for these experiments slightly acid to almost neutral (FRIEDMUTH & FISCHER 1958). On the other hand, the results also demonstrate that when the skin is damaged, the amount of boric acid absorbed is highly dependent on such factors as the nature of the vehicle and the size of the treated area.

Table 2

Amounts of boric acid (mg/kg body weight) applied and recovered in the urine. Mean values ± S. E. M. Treatment A: Preparation: 2 ml. Skin area: approximately 28 cm^2 *Condition of skin* severely damaged. Time: 8 hours.

Preparation	Rats	Applied	Boric acid				
			Recovered in the urine				
	no.	mg/kg	0–5 hours mg/kg	5–8 hours mg/kg	sum mg/kg	− control mg/kg	per cent of applied amount
I. Aqueous base (control)	7	–	1.2 ± 0.4	1.2 ± 0.3	2.4 ± 0.4	–	–
II. Boric acid jelly (about 2.5 % w/v)	15	290	32 ± 7	47 ± 6	98 ± 7 (P < 0.001)	96	33
III. Oleaginous base (control)	8	–	2.0 ± 0.4	0.9 ± 0.2	2.8 ± 0.5	–	–
IV. Boric acid/borate ol. ointment (about 2.8 % w/v)	8	340	4.3 ± 1.0	3.0 ± 0.6	7.2 ± 1.4 (P < 0.01)	4.4	1.5
V. *Complex* boric acid/borate ol. ointment (about 2.8 % w/v)	15	330	6.9 ± 1.2	6.0 ± 0.7	12.9 ± 1.4 (P < 0.001)	10	3

Table 3

Amounts of boric acid (mg/kg body weight) applied and recovered in both urine and preparation. Mean values ± S. E. M. Treatment B Preparation. 1 ml. Skin area 4.3 cm^2 Condition of skin: severely damaged. Time: 5 hours.

Preparation	Rats	Applied	Boric acid – Recovered after 5 hours – In preparation		In the urine		
	no.	mg/kg	mg/kg	per cent of applied	excreted mg/kg	– control mg/kg	per cent of applied amount
I. Aqueous base (control)	10	–	–	–	0.7 ± 0.1	–	–
II. Boric acid jelly (about 2.5 % w/v)	13	131 ± 1	33 ± 3	25	33 ± 4 ($P < 0.001$)	32 ± 4	24
III. Oleaginous base (control)	10	–	–	–	0.8	–	–
V. Complex boric acid/borate ol. ointment (about 2.8 % w/v)	12	150 ± 2	147 ± 1	98 (appr.)	2.1 ± 0.3 ($P < 0.001$)	1.3	1

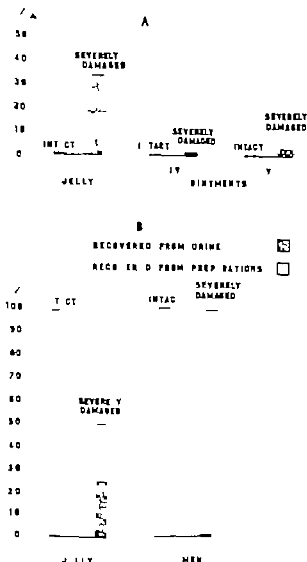


Fig. 2. All rats: Percentage recovery of boric acid. Mean values. Treatment A. Recovery from urine only (8 hours). Treatment B. Recovery from both urine and preparation (5 hours).

The vehicles used in the present experiments were chosen with the aim of comparing products actually in use, as for instance Sol. acidi borici Ph. Dan. 48 (purely hydrophilic) and natusan ointment (mainly hydrophobic). As a satisfactory application on rats of the boric acid solution was impossible, the jelly (II) was made to represent the hydrophilic preparation, and as natusan

Kent, N. L. & R. A. McCance: The absorption and excretion of 'minor' elements by man. *Biochem. J.* 1941, 35, 837–844.
Pfeiffer, C. C., L. F. Hallman & I. Gersh: Boric acid ointment. A study of possible intoxication in the treatment of burns. *J. Am. Med. Ass.* 1945, 128, 266–273.
Schou, J.: Borsyres giftighed specielt ved lokal anvendelse. *Arch. Pharm. Chem.* 1959, 66, 1069–1081.
Skipworth, G. B., N. Goldstein & W. P. McBride: Boric acid intoxication from "medicated talcum powder". *Arch. Derm.* 1967, 95, 83–86.
Svenningsen, Niels W.: Behandling av blöjdermatit. *Läkartidn.* 1966, 63, 2355–2359.
Wilken-Jensen, K.: Om behandling af bledermatitis. *Ugeskr. f. Læger* 1949, 37, 1035.

Acta pharmacol. et toxicol. 1970 28 425–434

From the Research Department,
N. V. Koninklijke Pharmaceutische Fabrieken v/h Brocades-Stheeman en Pharmacia,
Amsterdam, Holland

Toxicity Studies with Fluindarol, 2-[p-(trifluoromethyl)phenyl]-1,3-indandione, an Agent with Anticoagulant Properties (BS 7616 D)

By

C. J. van Eeken, R. D. R. Birtwhistle and D. Mulder*

(Received November 26, 1969)

Abstract. The phenylindandione derivative fluindarol was subjected to a toxicological investigation in view of its possible use as an anticoagulant in man. The acute oral LD50 in rats and rabbits was 198 and 123 mg/kg, respectively. The acute intraperitoneal LD50 in the rat was 125 mg/kg. Dogs survived single oral doses of up to 2810 mg/kg; if the drug was given for four consecutive days, an oral LD50 of 118 mg/kg was obtained. In a four week experiment in the rat, doses ranging from 108 to 7 mg/kg caused mortality which occurred sooner and more frequently in the females than the males; the organs showed haemorrhages and at the higher dose levels the liver showed parenchymal necrosis. Similar results were obtained with chlorindione, which tended to be somewhat less toxic. It was confirmed that distinction should be made between the "toxicity" of anticoagulant which is due to its pharmacological properties and true organ toxicity. Fluindarol was considered too toxic for clinical use.

Key-words: Anticoagulants – liver toxicity – phenylindandione derivatives.

During an investigation of a series of 2 aryl-1,3-indandiones, the p-trifluoromethylphenyl derivative, fluindarol, was found to possess a marked anticoagulant activity (*N. V. Kon. Pharm. Fabr. v/h Brocades-Stheeman en Pharmacia* 1966)*. A closer study of the compound showed that it was at least as active as the p-chlorophenyl analogue, clorindione, which is widely used as a drug in patients requiring antithrombotic treatment. Pharmacological screening revealed that, apart from a minor effect on the blood pressure fluindarol did not affect the circulation, respiration, CNS and cardiac activity and was devoid of any analgesic action.

---

* The pathological examinations were carried out by Professor Dr. R. Donner Institute of Pathology, Vrije Universiteit, Amsterdam, The Netherlands.

* N. V. Koninklijke Pharmaceutische Fabrieken v/h Brocades-Stheeman en Pharmacia, Brit. 1,041, 324, applic. August 27 1964, publ. December 29 1966.

In the present report a comparative toxicity study of fluindarol, as well as comparative studies with phenindione and clorindione is described. The toxicity of the latter compound has been described in detail by MONTIGEL & PULVER (1960)

Material and Methods

Fluindarol (fig. 1) occurs as a reddish-brown crystalline powder practically insoluble in water poorly soluble in methanol (1 230), and freely soluble in pyridine (1 5). The melting point is 180–183 and the molecular weight 290.2.

The toxicity was studied in the albino Wistar TNO rat, the mongrel dog, and the tame rabbit.

If not indicated otherwise, the drugs were administered in 1 % amylum suspension in concentrations corresponding to the dose per kg in 10 ml of the suspension.

Acute toxicity rat, oral route.

For the acute oral toxicity studies in the rat, equal numbers of young males and females (weight range 90–120 g) were divided at random into 6 groups of 8 animals each. The six logarithmically equidistant dose levels ranged between 100 and 350 mg/kg. After the animals had been fasted overnight and the drug administered via a gastric tube, they were observed for 7 days while receiving water and food *ad libitum*.

An analysis of regression was applied to find the LD50 and LD10, using log dose probit units (LITCHFIELD & WILCOXON 1949)

Acute toxicity rat intraperitoneal route.

We used the same 7-day test to evaluate the acute toxicity in rats of the same weight following intraperitoneal administration of fluindarol, the dose per kg was dissolved in 10 ml of 0.1 N sodium hydroxide solution (pH 12.5) Injection rate: 1 ml/min. The six logarithmically equidistant dose levels were between 100 and 200 mg/kg.

Acute toxicity rabbit oral route.

The acute oral toxicity in 11 tame rabbits (weight range 2.0–3.5 kg) was estimated by the use of the sequential staircase method (RÜMKE 1959). For the initial run we used 4 rabbits. The test compound was given via a gastric tube.

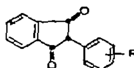


Fig. 1. Fluindarol, 2-[p-(trifluoromethyl)phenyl]-1,3-indandione.

R = H phenindione
R = CF_3 fluindarol
R = Cl clorindione
R = Br bromindione

Acute toxicity dog oral route.

We adopted the same test model for the determination of the acute oral toxicity in dogs (weight range 3.5–10.7 kg). Each animal received one dose of the compound in a gelatin capsule; if still alive at the end of a 7-day period, it was considered to have survived the experiment. The LD50 was calculated according to RÖMER (1959).

Cumulative toxicity dog oral route

The cumulative toxicity after dosage on 4 consecutive days was established in the same way.

Subacute toxicity rat oral route.

In a *4-week experiment* (I) each dose group consisted of 20 rats (10 females and 10 males), except for the 54 mg/kg and 27 mg/kg groups which consisted of 17 (female 10, male: 7) and 19 (female: 10, male: 9) animals, respectively. The starting weights ranged from 112–154 g for the female and 140–199 g for the male rats. The animals were weighed daily while the haemoglobin content, blood urea nitrogen level, number of erythrocytes and leucocytes per mm³ were determined and the differential counts were performed on the day before the experiment started and on the last day of the period. All the rats, both those which did not survive and the survivors, were autopsied. Fluindarol was administered via gastric tube 7 days a week, control group of 10 female and 10 male animals received an equal amount of the vehicle. The doses used were 108, 54 and 27 mg/kg, respectively. In an *additional 4-week experiment* (II) doses of 28, 14 and 7 mg/kg were given to groups of 14 (female: 7 male: 7), 14 (female: 7 male 7) and 12 (female: 6, male: 6) rats, respectively while control group numbered 12 rats (female. 6, male: 6). The starting weights ranged from 76–101 g for the female and 90–121 g for the mal rats.

In a *comparative study* with clorindione – duration 28 days – each dosage group consisted of 5 mal and 5 female rats; one group served as control. W checked the above parameters and determined SGPT and alkaline phosphatase values.

The dosage schemes included 54 27 and 13.5 mg/kg of fluindarol and these were compared with the equimolar doses of clorindione, being 49 24.5, and 12.25 mg/kg, respectively.

Moreover in *limited comparative experiment* we used 60 rats (weight range 90–120 g for the femal and 110–135 for the male rats) divided into 3 groups, which received fluindarol, clorindione, and phenindione respectively in doses of 35 mg/kg for 10 days. At the end of this period the dead animals in each group were counted.

In the subacute toxicity experiments the following organs from all the animals were subjected to gross and histopathological examination, heart, lungs, liver spleen, pancreas, genitals, stomach, intestine, thyroid, thymus, oesophagus, trachea, urinary bladder kidneys, adrenals, lymph nodes and salivary glands.

Results

Acute toxicity rat oral route

The acute oral LD50 was 198 mg/kg (161–244) The acute oral LD10 was 100 mg/kg (73–160) More females than males died. Only a few animals died on the day of administration Mortality was highest on the 3rd day after administration. None of the remaining rats died on the four last days.

19/20 confidence limits.

Acute toxicity rat intraperitoneal route

The acute intraperitoneal LD50 was 125 mg/kg (117–134)* The acute intraperitoneal LD10 was 113 mg/kg (103–124)* There was no difference in mortality between males and females. Death occurred on the 1st and 2nd day after administration.

Acute toxicity rabbit and dog oral route.

The acute oral LD50 in the rabbit was calculated at 123 mg/kg. After drug administration the animals refused any food but otherwise they did not appear to have been affected by the test compound. The rabbits which did not survive, died suddenly always during the night.

Attempts to determine the acute LD50 in the dog, failed. Doses up to 2810 mg/kg caused no mortality although the dogs vomited, refused food, and defaecated blood-stained mucus. The animals were inactive and remained lying in the cage. All of them, however stayed alive during the 7-day observation period.

Cumulative toxicity dog oral route.

The oral LD50 after administration for 4 consecutive days was 118 mg/kg. Although more pronounced, the toxicity pattern did not differ appreciably from that found after the administration of one single dose. The dogs showed no convulsions or tremors but became drowsy refused food, and lost a good deal of blood. A number of dogs in this series was autopsied. A large amount of blood was found in the intestine and colon all the organs were congested but distinct abnormalities were not encountered.

Subacute toxicity studies rat, oral route.

In the course of the *4-week experiment* 47 out of the 76 rats died. The distribution of the mortality over the various dose groups is given in table 1.

The mortality course revealed that females died sooner and in greater number than the males. The following signs were observed blindness, white ears, shallow and irregular respiration, and weakness. On account of the limited number of survivors it proved impossible to investigate whether the test compound had affected the growth in the doses given.

Blood tests showed serious anaemia (in some rats the number of erythrocytes was lower than 1 million/mm^3) and a marked decreased haemoglobin content in all the animals. As far as the determinations were possible, an increased urea nitrogen level and of burr-shaped erythrocytes were found in the highest dose groups in both sexes. On pathological examination a conspicuously large number of rather extensive haemorrhages were observed in the

---

19/20 confidence limits.

Table 1

Mortality in rats during two 4-week experiments with fluindarol.

Exp.	Dosage mg/kg/day	Number of animals that did not survive/ Number of animals	
		Female	Male
I	108	10/10	10/10
	54	10/10	6/7
	27	7/10	5/9
	0	0/10	0/10
II	28	6/7	2/7
	14	3/7	0/7
	7	2/6	1/6
	0	0/6	0/6

subcutaneous tissue and various organs. The liver showed not only foci of infiltration but also foci of parenchymal cell damage and parenchymal necrosis.

In the *additional experiment* 14 rats died the distribution of mortality is given in table 1 Again, there was a distinct difference in mortality between males and females. The growth of the survivors did not appear to be affected by the test compound. Macro- and microscopic examinations revealed petechiae on the pleura in the rats of all the dose groups, and also in this respect there was a clear difference: the number of petechiae was higher in the females.

Comparative study of fluindarol and clorindione.

Mortality occurred in all the dose groups. After 12 days all the rats receiving the highest dose of fluindarol had died. None of the rats treated with 49 mg/kg of clorindione survived for more than 24 days.

In the next lower dose groups, 80 / of the fluindarol group did not survive a 28-day period of administration while in the same period of time an equimolar dose of clorindione resulted in a mortality of 70 /

In the lowest dose groups the percentages of survivors were 50 / for both drugs (table 2)

It can be concluded that the rats treated with fluindarol died within a shorter time than the rats given clorindione.

In both the fluindarol and the clorindione groups the same phenomena were observed, viz. loss of weight one or two days before death, indications of blindness, white ears and extremities, loss of blood from the nose and uri tract, haemorrhages in the neck and the extremities, and general w

Table 2

Mortality in rats treated with equimolecular amounts of fluindarol and clorindione for 28 days.

Dose mg/kg/day	Fluindarol Females %	Males %	Dose mg/kg/day	Clorindione Females %	Males %
54	100	100	49	100	100
27	100	60	24.5	80	60
13.5	80	20	12.25	60	40
0	0	0			

A peculiarity observed in most of the animals treated with clorindione was that the hair was more yellow than under normal conditions.

Again, the above phenomena occurred first in the fluindarol groups. The rapid onset of mortality however made it practically impossible to complete the blood tests. From the available data it appears, however that even a few doses of either compound caused anaemia and a reduced haemoglobin level, first in the fluindarol treated groups and then in the clorindione groups.

The determinations of the blood urea nitrogen level and differential counts revealed abnormalities in the groups which received the highest and medium doses of fluindarol. In the 54 mg/kg group a blood urea nitrogen level of 72 mg / was found in one case and in the 27 mg/kg group values of up to 180 mg / occurred.

The differential count showed the presence of myeloblasts, erythroblasts, polynuclear erythroblasts with 2–10 nuclei, and burr-shaped erythrocytes. In the animals examined, the SGPT and alkaline phosphatase values showed a tendency to rise rapidly in the days preceding death. Due to the progressive mortality in all the dose groups it was impossible to determine whether this had any effect on growth at the end of the experiment.

All animals were autopsied and microscopically examined. Haemorrhages were found in the various organs of most rats, haemothorax was also frequently observed. In the livers of the animals which had not survived the experiment, foci of necrosis were found. The presence of these foci was distinctly dose-related, incidence and intensity were highest in the groups treated with the largest doses. The highest doses – especially of fluindarol – resulted in marked necrosis. At the lowest dose level however clorindione caused more liver necrosis than fluindarol.

Thymus haemorrhages were often found, though more frequently in the clorindione groups.

In the rats that had received the medium and lowest doses of clorindione, many pancreatic haemorrhages were observed. None of the above abnormalities were observed in the control animals.

Limited comparative experiment

In the study of the oral toxicities of the three related compounds phenindione given in doses of 35 mg/kg/day proved to be the least toxic, since none of the animals to which it had been administered, died. Clorindione was lethal in 10 animals (50 /) and fluindarol caused the death of 8 animals (40 /) during the period of study

Discussion

It is well established that small changes in the 2-phenyl-1,3-indandione (phenindione) molecule may have a profound effect on its toxic and pharmacological activities (MONTIGEL & PULVER 1960 FONTAINE *et al.* 1967) Generally speaking, *para* substitution in the 2-phenyl group results in compounds that are more active than the parent compound (VIGRAN 1967), the halogen-substituted derivatives being especially powerful anticoagulants (O CONNOR *et al.* 1952, KIENEL 1968). The action of the *p*-bromo compound (bromindione) for example, is 14–20 times as strong as that of phenindione itself (STROBEL 1962). Moreover in a number of cases the toxicity of the substituted compounds is reduced as compared with the parent compound, so that effects of substitution seem to be very specific (see also RENK & STOLL 1968)

Although the pharmacological activity and low toxicity of phenindione are well known (O'CONNOR *et al.* 1952), this anticoagulant has been reported as not being entirely harmless (MED. LETTER 1963). As is shown in table 3 the acute oral toxicity of fluindarol, which is a highly active anticoagulant (at least as active as clorindione), is higher than that of phenindione or clorindione.

The acute LD50 of fluindarol established in our experiments are also lower

Table 3

LD50 values for phenindione and its trifluoromethyl and chloro derivatives.

Compound	Species	Route	LD50	(19/20 conf. limits)	Ref.
Fluindarol ...	rabbit	oral	123		
	rat	oral	198	(161 244)	
	rat	i. p	125	(117–134)	
Phenindione	rat	oral	360	(286–454)	KOVALČÍK *et al.* (1959)
	rat	oral	175		MONTIGEL & PULVER (1960)
	rat	oral	240	(165–348)	
	mouse	oral	175		MONTIGEL & PULVER (1
Clorindione	rat	oral	45	(168–355)	

than the values reported by KOVALCIK *et al.* (1959) for the nitronaphthyl derivative, and by CORRELL *et al* (1952) for the 2-diphenylacetyl derivative.

TUMANOVSKII *et al* (1965) studied the toxicity of 26 derivatives of 1,3-indandione but did not publish the results for the two sexes separately

Previous investigators also found differences in the toxicity of anticoagulants between male and female animals. VENHO (1959) found that on administration of phenindione to mice fewer females than males died. His experiments with castrated and hormone treated mice indicated that the toxicity pattern of anticoagulants is distinctly affected by the sex hormones. Our results also show a sex difference in rats, but in the opposite direction. For this difference we have no explanation apart from the possibility of species-linked characteristics.

Although the literature affords comparatively little information on the toxicity pattern produced by long-term administration of 2 arylindandiones, it is clear that the ideal anticoagulant has not been found in the indandione series. One of the major objections to oral anticoagulants is their cumulative toxic effect. Fluindarol is not an exception and neither are the differently substituted analogues although there appear to be differences in degree (KOVALCIK *et al* 1959 KABAT *et al* 1944). According to O CONNOR (1952) even phenindione itself, which is widely considered to be a non-toxic anticoagulant is not devoid of cumulative toxicity

The existing diversity of opinion is further illustrated by the following discrepancies. whereas the cumulative toxicity of phenindione is regarded to be less than that of the oxycumarins – among which we also observed cumulation (MULDER & VAN EEKEN unpublished) some authorities do consider that the phenylindandiones are more harmful than the oxycumarins (INGRAM 1961 MED. LETTER 1963) According to LASAGNA (1965) the anticoagulants of the phenindione type should certainly be regarded as being far from harmless if only because of their haemopoetic, renal and hepatic toxicity

The mortality pattern is often taken as a measure of cumulation. It is, however uncertain whether this criterion is applicable in toxicity experiments with anticoagulants (MONTIGEL & PULVER 1960) Many investigators believe that two different criteria should be applied the deaths due to haemorrhages (a) and side effects other than haemorrhages (b) the latter group determining the real toxicity In this respect there is some difference between our observations regarding clorindione and fluindarol and the results of Jaques experiments (JAQUES *et al.* 1950) with phenindione in dogs and rabbits apart from a mild fatty degeneration in the limbs of the Henle loops in the kidney Jaques found no pathologic phenomena in most of the organs. In our investigation both fluindarol and clorindione were found to have a distinctly necrotizing effect on the liver a phenomenon not due to

the haemorrhages. Moreover fluindarol as well as clorindione induce haemorrhages in the thymus, whereas according to Jaques the administration of 8.3 mg/kg of phenindione on 48 consecutive days to dogs did not induce purely toxic effects.

A 6-week toxicity study with bromindione in guinea pigs (*U S Vitamin and Pharmaceutical Corp* 1962)* is reported to have shown no other abnormalities but a significant increase in liver weight, while on the other hand the bromo derivatives as a rule are more active than the chloro derivatives. Hence in the phenindione series, bromo substitution would seem to result in a wider safety margin than chloro substitution.

According to KOVALCIK *et al* (1956) the bromo and chloro derivatives of 2-(1-naphthyl)-1, 3-indandiones are also less toxic in the rat than the parent compound.

The toxic pattern shown by fluindarol in our series of experiments is, in our opinion, at least partly attributable to the trifluoromethyl group. We have reported elsewhere (VAN EEKEN & MULDER 1966) on the toxicity of other compounds carrying this group

The problem which still remains is the reason for the rather marked cumulative toxic effect which all phenindione derivatives have in common. BURNS & DESMOND (1968) ascribe this toxicity to the presence of the modified benzene ring in the compound, and the side effects which MENON (1968) encountered in his studies led him to come to a similar conclusion.

Our simultaneous, comparative study with phenindione and fluindarol and clorindione showed that at the same dose level the first compound is markedly less lethal than the other two compounds.

These results, in combination with the data in the literature, clearly indicate that fluindarol should not be admitted for clinical trial. They also make it clear that in a toxicity investigation on anticoagulants it is particularly important to differentiate between the effects of the intrinsic pharmacological properties – mortality or pathological changes due to haemorrhages – and the toxic effects on organs such as the liver and kidneys.

Acknowledgement

The authors thank Mr F Haandrikman, Mr L. G M. Kenter and Mr P van der Kwast for their excellent technical assistance in the experiments.

REFERENCES

Burns, Ch. & F B. Desmond. Sensitivity to dindevan (phenylindanedione). Report of a case with review of the literature. *New Zealand Med. J* 1968, 57 283–291.

U S. Vitamin and Pharmaceutical Corporation: Ger 1,130,439 May 30, 1962, U S. Applic. June 27 1960.

Correll, J T L. L. Coleman, St. Long & R. F Willy Diphenylacetyl-1,3-indandione as a potent hypoprothrombinemic agent. *Proc. Soc. Exptl. Biol. Med* 1952, 80, 139–143.

Eeken, C. J van & D Mulder Toxicological properties of *N N*-dimethyl-4-phenyl-4-(α, α, α-trifluoro-*p*-tolyl)-3-butenylamine hydrochloride (BS 7308). *Arch. Int Pharmacodyn* 1966, 160 231–240.

Fontaine, L., M. Grand, D Molho & E. Boschetti. Activité anti-inflammatoire expérimentale de coumarines, indane-diones et acyl-indane-diones apparentées aux anticoagulants oraux. *Med Pharmacol. Exptl.* 1967 17 497 507

Ingram, G I. C. Anticoagulant therapy *Pharmacol. Rev* 1961, 13, 279–328.

Jaques, L. B., E. Gordon & E. Lipp A new prothrombopenic drug, phenylindanedione. *Can. Med Assoc. J* 1950, 62, 465–470 Ref. from *Chem. Abstr* 1951, 45, 76

Kabat, H., E. F Stohlman and M. I. Smith. Hypoprothrombinemia induced by administration of indandione derivatives. *J Pharmacol.* 1944 80 160–170.

Kienel, G Halogenierung und embryotoxische Wirkung. *Arzneimittelforsch.* 1968, 18, 658–661.

Kovalčík, V J Pecháň, V Smiško & V Fischerová. Zu den Eigenschaften der neuen Derivate der Indandionantikoagulantien. *Arch. Exptl. Pathol Pharmakol.* 1959 236, 288–289

Kovalčík, V J Valach, J Pecháň & P Hrnčiar Anticoagulant effect of new indanedione derivatives. *Ceskoslov Farmacol.* 1956, 5, 391–395 Ref. from *Chem. Abstr* 1960, 54, 11275a

Lasagna, L. The diseases drugs cause. In. *Drug-induced diseases*, 2nd Symposium Boerhaave courses for post-graduate medical Education, p. 1, 1965, Int. Congr Ser no. 85, Excerpta Medica Foundation, Amsterdam.

Litchfield, J T Jr & F Wilcoxon. A simplified method of evaluating dose-effect experiments. *J Pharmacol.* 1949 96 99–113.

Med. Letter 1963 6, 63.

Menon, I S. Side-effects of phenindione. *Brit. Med J* 1968, II, 622.

Montigel, C. & R. Pulver G25766, ein Derivat des Phenylindandions mit antithrombotischer Wirkung. *Schweiz. Med. Wochenschr* 1960, 90, 213–217

O'Connor Wm. R., Ch. E. Thompson & L. A. Baker Experience with the new anticoagulant phenylindanedione in acute myocardial infarction. *Quart Bull. N W Univ Med. School* 1952, 26, 193–196 Ref. from *Chem. Abstr* 1952, 46, 10448

Renk, E. & W G Stoll. Orale Antikoagulantien. *Fortschr Arzneimittelforsch.* 1968, 11, 226–355

Rümke, Chr L. The influence of atropine on the toxicity of thialbarbitone, thiopentone and pentobarbital (With an appendix on sequential toxicity testing). *Arch. Int. Pharmacodyn.* 1959 119 10–19

Strobel E. Klinische Untersuchungen über die gerinnungshemmende Aktivität eines neuen Antikoagulans der Indandion-Reihe. *Arzneimittelforsch.* 1962, 12, 718–720.

Tumanovskii, M. N., V I Zavrazhnov & V B. Pokl. Experimentelle und klinische Anwendung neuer gerinnungshemmender und gerinnungsfördernder Mittel. *Z. Ges. Inn. Med.* 1965, 20 Ber (63) 328–329

Venho, E. V Pharmakologische Untersuchungen über die Toxizität und die Antagonisten der synthetischen Antikoagulantien. *Ann. Med. Exptl. Biol. Fenn.* 1959 37 Suppl. 6, 1–76.

Vigran, I. M. Further studies of bromindione, a newer anticoagulant. *Curr Ther Res.* 1967 9 372–378.

Acta pharmacol. et toxicol. 1970, 28 435–444

From the Toxicology Laboratories, AB Astra, Södertälje, and
Research Laboratories, AB Hässle, Gothenburg, Sweden

Renal Lesions Induced by α Methyltyrosine Methylester and α Methyltyrosine

By

Göran Magnusson, Hans Corrodi and Eskil Hansson

(Received February 27 1970)

Abstract. The toxicity of α-methyltyrosine methylester (H 44/68) and α-methyltyrosine (H 9/88) in the rat was investigated in four week subacute study Both compounds induced renal lesions with formation of calculi in the urinary tract. The renal lesions were manifested by deposition of tubular crystals associated with regressive and inflammatory changes. The main sites of crystal deposition were the collecting tubules and the papillary ducts. The calculi consisted of α-methyltyrosine. The investigation indicates that α-methyltyrosine methylester is metabolized in the body to the less soluble α-methyltyrosine and that α-methyl tyrosine is excreted through the kidneys. Crystals of α-methyltyrosine are formed in the renal tubules and urinary tract probably due to supersaturation of the urine.

Key-words: Methyltyrosine – toxicity

α-Methyltyrosine and its methylester have the capacity to inhibit tyrosine hydroxylase (SPECTOR *et al.* 1965 WEISSMAN & KOE 1965 MAITRE 1965 HANSON 1965 CORRODI & MALMFORS 1966 ANDÉN *et al.* 1966) Due to this property the compounds have been used as tools for the study of the sympathetic nervous system.

However MOORE *et al.* (1967) have shown that the interpretation of the results of some of the studies with α-methyltyrosine might be complicated by the toxicity of the drug α-Methyltyrosine has been shown to induce renal damage in the rat (MOORE *et al* 1967) and to produce crystalluria and urolithiasis in the dog (ENGELMAN *et al* 1968b). The pathogenesis of the renal damage in the rat is not clear (MOORE 1967 HOOK & MOORE 1969)

The related compound 3-α-dimethyltyrosine methylester (H 59/64) also acts as a potent inhibitor of tyrosine hydroxylase (HANSON 1967) This compound has been shown to cause renal lesions with the formation of
in the urinary tract (MAGNUSSON *et al.* 1968).

The present investigation was conducted in order to elucidate whether the mechanism of the renal lesions caused by α-methyl-tyrosine (H 9/88) was of the same nature as previously demonstrated for 3-α-dimethyltyrosine methylester. The effect of α-methyltyrosine methylester hydroclonde (H 44/68) was also studied. This compound has the same pharmacological action as α-methyltyrosine. It is, however, easily water soluble and it was therefore of interest to see whether this property would change the renal toxicity of the drug.

Material and Methods

A total of 35 approximately two month old male rats of the Sprague-Dawley strain were used in the experiments. The animals were given food and water ad libitum. The rats were divided into seven groups, each consisting of five animals. Data on the different groups and treatments are given in table 1. α-Methyltyrosine and α-methyltyrosine methylester HCl were supplied by the Research Laboratories, AB Hässle, Gothenburg, Sweden. α-Methyltyrosine was suspended in 2 per cent methyl cellulose in a concentration of 200 mg/ml and α-methyltyrosine methylester HCl was dissolved in distilled water to concentration of 200 mg/ml.

The rats were treated once a day for four weeks. The compounds were always given by gavage. During the experiment the rats were observed daily for any clinical signs. Before the start and as a rule once a week during the experiment blood samples were taken for examination of blood urea-nitrogen (UN Test® Hyland Div. Travenol Laboratories, Inc., Los Angeles, California, U.S.A.). At the end of the experiment the rats were sacrificed. Autopsies were performed on all the rats, including those which died, and the kidneys were examined microscopically.

Samples of the kidneys were fixed in 10 % formalin solution, and in Bouin's and Carnoy's fixative fluid. Paraffin embedded sections were stained with haematoxylin and eosin, van Gieson and periodic acid Schiff's reagent (PAS). Frozen sections were stained with Sudan III for examination of fat.

Identification of α-methyltyrosine in the urinary calculi was performed as follows: The calculi were dissolved in the least possible amount of 1 N-HCl. The amino acid was precipitated by saturated sodium acetate solution. The amino acid was filtered, washed with water and dried. Identification was done by means of the IR-spectrum (in KBr) which was identical with that of authentic dl-α-methyltyrosine. The melting point was > 250° with decomposition and there was no depression of the melting point after mixture with the authentic substance.

Result

Clinical signs

At a high dose level of both α-methyltyrosine methylester and α-methyltyrosine the rats showed a decreased body weight gain and a poor general condition with piloerection. A total of fourteen rats died or were killed in a moribund state during the experiment. Of the animals given α-methyltyrosine methylester four rats died within 9–14 days after treatment with 600 mg/kg and all five rats died within seven days after treatment with 1000 mg/kg. The five rats given 1000 mg/kg of α-methyltyrosine died within 13–21 days (table 1).

Table 1

Four week subacute toxicity studies in rats with α-methyltyrosine methylester (H 44/68) and α-methyltyrosine (H 9/88).

Group number	Treatment	Dose (mg/kg)	Concentration of solution (mg/ml)	Number of days of treatment
1	No treatment	Control		28
2	α-methyltyrosine methylester*	200	200	28
3		600	200	9–28
4		1000	200	7
5	α-methyltyrosine*	200	200	28
6		600	200	28
7		1000	200	13–21

* α-methyltyrosine methylester was dissolved in water
** α-methyltyrosine was suspended in 2 % methyl cellulose.

Laboratory investigations

An increase in the urea-nitrogen of the blood serum was demonstrated in three groups, 600 mg/kg and 1000 mg/kg of α-methyltyrosine methylester and 1000 mg/kg of α-methyltyrosine (fig. 1). The highest values were about 240 mg/100 ml and these occurred following a dose of 1000 mg/kg of α-methyl tyrosine methylester

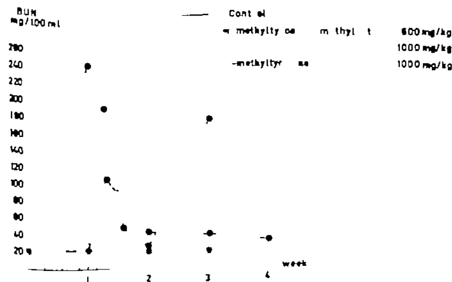


Fig 1. Concentration of urea-nitrogen in blood serum (BUN) of rats after tion of α-methyltyrosine methylester and α-methyltyrosine. As rule, each poi mean of five values. ● indicates the day of sampling.

Table 2

Kidney weights in rats after administration of α-methyltyrosine methylester and α-methyltyrosine.

Treatment	Dose (mg/kg)	Number of animals	Kidney weight: Mean weight in grams (range in brackets)	Kidney weight: Per cent of body weight (mean value)	Kidney weight: Number of rats with renal lesions
No treatment	Control	5	2.56 (2.43–2.75)	0.69	0
α-methyltyrosine methylester	200	5	2.37 (2.30–2.49)	0.64	0
	600	5	2.41 (2.18–2.83)	0.83	5
	1000	5	2.56 (2.26–3.59)	1.00	5
α-methyltyrosine	200	5	2.40 (2.10–2.80)	0.76	0
	600	5	2.42 (2.00–2.76)	0.74	2
	1000	5	2.34 (2.17–2.75)	0.92	5

Gross pathology

Pathological changes in the kidneys were observed in all rats treated with 600 and 1000 mg/kg of α-methyltyrosine methylester and with 1000 mg/kg of α-methyltyrosine. The kidneys were enlarged (table 2) had a pale colour a somewhat firm consistency and a fine granular surface. The cut surface of the kidneys was moist and showed white foci about 0.5 mm in size. These white foci were more numerous in the medulla than in the cortex.

White round or irregularly shaped firm calculi up to 5 mm in size were observed in the renal pelvis, ureters and/or urinary bladder (fig. 2). Such calculi occurred after treatment with α-methyltyrosine methylester in four



Fig. 2. Urinary calculi in a rat after administration of α-methyltyrosine for 28 days. Magnification × 5

rats given 600 mg/kg and in two rats given 1000 mg/kg after treatment with α-methyltyrosine calculi were found in one rat given 600 mg/kg and in five rats given 1000 mg/kg. A uraemic gastro-enteritis was observed in those rats which died.

Microscopic pathology

Lesions of the kidneys were observed in all the rats given 600 and 1000 mg/kg of α-methyltyrosine methylester and in two rats given 600 mg/kg of α-methyltyrosine and in all the rats given 1000 mg/kg of α-methyltyrosine (table 2). In the other treated and control animals the kidneys showed a normal picture. The renal lesion had the same appearance in all the rats.

The renal tubules contained crystals of various sizes. The crystals were concentrated in the collecting tubules and papillary ducts (fig. 3) They were round or oval in shape and had a homogenous or somewhat filamentous pattern. The edges of the crystals had a smooth appearance. Several tubular lumens were occluded by the crystals (fig. 4) The crystals were eosinophilic and showed a PAS-positive reaction. Proximal to the crystals Bowman's spaces were enlarged and the renal tubules were dilated with flattening of the epithelium (fig. 5) The epithelium of the proximal convoluted tubules showed a slight or moderate fine droplet fatty change The tubular epithelium



Fig. 3. Deposition of crystals in the tubules of the renal papilla in rat after administration of 1000 mg/kg of α-methyltyrosine methylester for seven days. Hematoxylin and eosin. Magnification × 100.

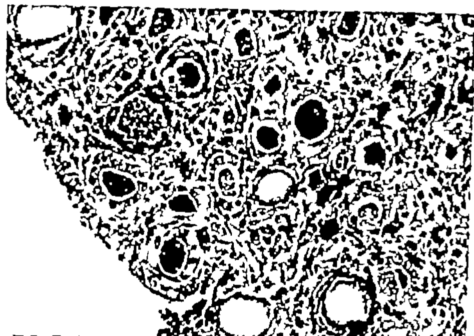


Fig. 4 Crystals occluding tubular lumens necrobiotic nuclear changes of the tubular epithelium, and casts in the tubules of the renal papilla in a rat after oral administration of 1000 mg/kg of α-methyltyrosine methylester for seven days. Haematoxylin and eosin. Magnification × 50.

displayed necrobiotic nuclear changes and necrosis. The lumens contained granulocytes, desquamated epithelium, and necrotic cells, forming casts (fig. 6) The crystals were surrounded by inflammatory cells. In the interstitial tissue there were inflammatory changes with infiltration of granulocytes, lymphocytes, and histiocytes (fig. 7). A slight interstitial fibrosis was present.

The lesions of the kidneys were found in both the medulla and the cortex. The changes were, however much more severe in the medulla, above all in the renal papilla, than in the cortex. The lesions had a focal distribution in the cortex and an almost diffuse distribution in the renal papilla. The alterations in the papilla were most evident in the rats which died within fourteen days after the start of the experiment. In the two rats given 600 mg/kg of α-methyltyrosine the renal damage was slight. In the other rats the changes were considered to be of a moderate or severe degree.

Chemical investigations.

The urinary stones consisted of α-methyltyrosine after treatment with both α-methyltyrosine and α-methyltyrosine methylester HCl

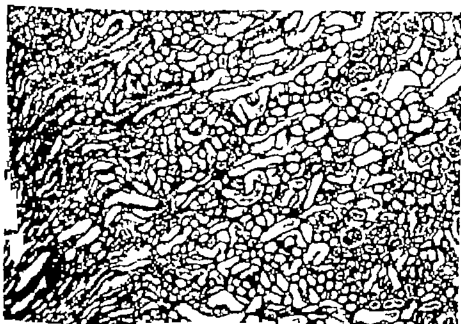


Fig. 5. Dilatation of the tubules in rat kidney after oral administration of 1000 mg/kg of α-methyltyrosine methylester for seven days. Haematoxylin and eosin. Magnification × 40

Discussion

Studies of the metabolic fate of orally administered α-methyltyrosine in animal and man has shown that it is excreted mainly unchanged. In man less than 1 / of the administered drug could be recovered in the urine as catechol metabolites (ENGELMAN *et al* 1968a)

The main factors involved in the renal handling of α-methyltyrosine in rats are glomerular filtration and reabsorption (HOOK & MOORE 1969) In the present investigation the identification of α-methyltyrosine as a constituent of the urinary calculi confirms the excretion of α-methyltyrosine via the kidneys. The present study also shows that α-methyltyrosine is the main component in the urinary calculi after administration of α-methyltyrosine methylester

The gross and microscopic examinations revealed that α-methyl-tyrosine causes injury to the kidneys with formation of calculi in the urinary tract. The damage was characterized by deposition of crystals in the renal tubules, alterations in the tubular epithelium and inflammatory changes. The renal changes should be interpreted as a primary tubular lesion. The deposition of crystals was concentrated to the collecting tubules and papillary ducts. The predilection for this site of precipitation could be related to
of α-methyltyrosine due to the progressive concentration of the urine



Fig. 6. Occasional crystals and numerous casts in dilated tubules of the renal papilla in a rat after oral administration of 1000 mg/kg of α-methyltyrosine for 21 days. Haematoxylin and eosin. Magnification × 100.

tubules. The frequency of renal lesions was very similar between rats treated with α-methyltyrosine and its methylester. It is therefore likely that the hydrolysis of the latter compound to α-methyltyrosine is a rapid and efficient process.

The dilatation of the renal tubules and the enlargement of the kidneys are explained by a stasis of the urine flow due to an occlusion of the tubular lumens by the crystals. An increased intratubular hydrostatic pressure gives rise to an insufficient filtration in the glomeruli which is reflected by the increase in the blood urea-nitrogen. The cause of death is a uraemia, as indicated by the high values of urea-nitrogen and by the occurrence of uraemic gastro-enteritis.

The great variation in the blood urea-nitrogen values observed in our study is probably due to an irregular water intake. It is known that in conditions in which deposits of calculi in the renal tubules occur a great water intake decreases the precipitation of crystals and the values of blood urea-nitrogen (Moore *et al.* 1967, Magnusson *et al.* 1968). Water diuresis is also used in



Fig. 7 Chronic interstitial inflammatory changes of the renal cortex in a rat after oral administration of 600 mg/kg of α-methyltyrosine methylester for 28 days. Haematoxylin and eosin. Magnification × 100.

the treatment of urolithiasis, e. g. in cystinuria (HAMBRAEUS & BOSTRÖM 1960).

Deposits of any crystals can display different locations in the renal tubules. The closely related compound 3 α-dimethyltyrosine as well as sulphonamides form calculi in the collecting tubules and papillary ducts in a manner similar to α-methyltyrosine (MAGNUSSON *et al.* 1968 SMITH & JONES 1961) Calcium oxalate crystals due to intoxication with ethylene glycol, are an example of deposits mainly located in the proximal renal tubules (JÖNSSON & RUBARTH 1967 ROBERTS & SEIBOLD 1969).

Kidney lesions were demonstrated by MOORE *et al.* (1967) in toxicity studies with α-methyltyrosine. As possible causes of the renal lesions MOORE *et al* (1967) considered mechanical damage to the epithelium, effects on the cells during reabsorption or secretion, and changes in the renal blood flow In the present investigation the deposits of crystals occluding the tubular lumens were restricted to the renal medulla and papilla. In these parts of the kidneys the urine is most saturated. The most severe lesions also appeared in these areas of the kidneys. Thus these conditions suggest that the mechanical damage by the crystals is the most essential factor in the pathogenesis of the renal lesion by α-methyltyrosine.

No urinary calculi were observed in the studies by MOORE *et al* (1

contrast to the present study MOORE *et al* (1967) administered 200 and 300 mg/kg intraperitoneally to rats by single injections while the present study is concerned with repeated administration of much higher oral doses. In dogs crystalluria and urolithiasis have been observed after the administration of α-methyltyrosine in doses as small as 50 mg/kg/day However no evidence of urolithiasis has been found in patients receiving up to 4 g a day and in contrast to human subjects, dogs that developed evidence of crystalluria and stones ate and drank poorly (ENGELMAN *et al*, 1968b). The *in vitro* solubility of α-methyltyrosine in aqueous solution is very low and it is therefore evident that the formation of calculi must be related to dose levels and water intake.

REFERENCES

Andén, N E., H. Corrodi, H. Dahlström, K. Fuxe & T Hökfelt: Effects of tyrosine hydroxylase inhibition on the amine levels of central monoamine neurons. *Life Sciences* 1966, 5, 561–568.

Corrodi, H. & T Malmfors: The effect of nerve activity on the depletion of the adrenergic transmitter by inhibitors of noradrenaline synthesis. *Acta Physiol. Scand* 1966 67 352–357

Engelman, K., E. Jequier S. Udenfriend & A. Sjoerdsma. Metabolism of α-methyltyrosine in man. Relationship to its potency as an inhibitor of catecholamine biosynthesis. *J Clin. Invest* 1968a, 47 568–576.

Engelman, K., D. Horwitz, E. Jequier & A. Sjoerdsma. Biochemical and pharmacologic effects of α-methyltyrosine in man. *J Clin. Invest* 1968b, 47 577–594

Hambraeus, L. & B. Boström. Cystinuri. *Svensk Läk. Tidn.* 1960, 57 3159–3173.

Hanson, L. The disruption of conditioned avoidance response following selective depletion of brain catechol amines. *Psychopharmacologia* (Berl.) 1965, 8, 100.

Hanson, L. Biochemical and behavioural effects of tyrosine hydroxylase inhibition. *Psychopharmacologia* (Berl.) 1967 11 8–17

Hook, J B. & K. E. Moore: The renal handling of α-methyltyrosine. *J Pharmacol. exp Ther* 1969 168 310–314

Jonsson, L. & S. Rubarth Ethylene glycol poisoning in dogs and cats. *Nord. Vet Med.* 1967 19 265–276

Magnusson, G H. Corrodi & E. Hansson. Renal lesions induced by 3, α-dimethyltyrosine methylester HCl. *Proc. European Society Study Drug Toxicity* Xth Meeting, Oxford, April 1968, 206–217

Maître, L. Presence of α-methyl-dopa metabolites in heart and brain of guinea pigs treated with α-methyl-tyrosine. *Life Sciences* 1965 4 2249

Moore, K. E., P F Wright & J K. Bert. Toxicological studies with α-methyltyrosine, an inhibitor of tyrosine hydroxylase. *J Pharmacol. exp Ther* 1967 155 506–515.

Roberts, J A. & H. R. Seibold. Ethylene glycol toxicity in the monkey *Toxicol. appl. Pharmacol.* 1969 15, 624–631.

Smith, H. A. & T C. Jones. *Veterinary pathology* Lea & Febiger Philadelphia, Pa., 1961, p. 626

Spector S., A. Sjoerdsma & S. Udenfriend. Blockade of endogenous norepinephrine synthesis by α-methyl-tyrosine, an inhibitor of tyrosine hydroxylase. *J Pharmacol. exp Ther* 1965 147 86

Weissman, A. & B. K. Koe: Behavioral effects of L-α-methyltyrosine, an inhibitor of tyrosine hydroxylase. *Life Sciences* 1965, 4, 10.

Acta pharmacol. et toxicol. 1970 **28**, 445–453

From the Research and Development Laboratories, Astra Pharmaceuticals, Södertälje, Sweden

Stereospecificity of the Enzymatic Biotransformation of the Enantiomers of Prilocaine (Citanest®)

By

B. Åkerman and S. Ross

(Received March 19 1970)

Abstract: No differences have been found between D-(−)- L-(+)- and DL-(±)-prilocaine (citanest®) with regard to intravenous LD50's in mice, nor have any differences been found between the D-(−)- and L-(+)-forms on subcutaneous injection. Compared to L-(+)-prilocaine, D-(−)-prilocaine was less toxic on intravenous infusion (mice, 0.12–0.30 mg/min.) and had less cumulative effect in rabbits (20 mg/kg each 15 min.). The plasma concentrations (cats) of the D-(−)-form (25 mg/kg intravenously) were lower than those of the L-(+)-form and more *o*-toluidine, one of the metabolites of prilocaine, was detected in the blood. D-(−)-prilocaine also produced methaemoglobinaemia at a more rapid rate. The ^{14}C-labelled enantiomers of prilocaine were hydrolyzed at significantly different rates by liver homogenates, slices (mice, rabbits, cats) and isolated microsomes (rabbits). D-(−)-prilocaine had the highest affinity for the enzyme(s). Thus, a significant stereospecificity of the hydrolyzing enzymes in their reaction with the enantiomers of prilocaine was found. However no evidence was obtained which could suggest substitution of the local anaesthetic prilocaine by either of its optical isomers.

Key-words: Anaesthetics, local – metabolism optical isomers – prilocaine.

Recently there has been a growing interest in the relative effects of the enantiomers of local anaesthetic drugs. Evidence has been produced, showing that the enantiomers of aminoacylanilides may diverge significantly with regard to their local anaesthetic properties (ÅKERMAN *et al.* 1967 LUDUENA 1969 ADLER *et al* 1969). In addition to having practical consequences, such findings may also throw some light on the complexity of factors determinin local anaesthetic potency The results of studies on enantiomers of spir chimides (ÅKERMAN *et al.* 1969 ÅKERMAN & SOKOLL 1969) are even interesting from a theoretical point of view since they indicate of sensitivity to steric factors in the actual blocking mechanism.

contrast to the present study MOORE *et al.* (1967) administered 200 and 300 mg/kg intraperitoneally to rats by single injections while the present study is concerned with repeated administration of much higher oral doses. In dogs crystalluria and urolithiasis have been observed after the administration of α-methyltyrosine in doses as small as 50 mg/kg/day However no evidence of urolithians has been found in patients receiving up to 4 g a day and in contrast to human subjects, dogs that developed evidence of crystalluria and stones ate and drank poorly (ENGELMAN *et al* 1968b) The *in vitro* solubility of α-methyltyrosine in aqueous solution is very low and it is therefore evident that the formation of calculi must be related to dose levels and water intake.

REFERENCES

Andén, N E., H. Corrodi, H. Dahlström, K. Fuxe & T Hökfelt. Effects of tyrosine hydroxylase inhibition on the amine levels of central monoamine neurons. *Life Sciences* 1966, 5 561–568.

Corrodi, H. & T Malmfors: *The effect of nerve activity on the depletion of the adrenergic transmitter by inhibitors of noradrenaline syntheses. Acta Physiol. Scand* 1966, 67 352 357

Engelman, K., E. Jequier S. Udenfriend & A. Sjoerdsma. Metabolism of α-methyltyrosine in man Relationship to its potency as an inhibitor of catecholamine biosynthesis. *J Clin. Invest.* 1968a, 47 568–576.

Engelman, K., D Horwitz, E. Jequier & A. Sjoerdsma. Biochemical and pharmacologic effects of α-methyltyrosine in man. *J Clin Invest.* 1968b, 47 577–594.

Hambraeus, L. & B Boström. Cystinuri. *S ensk Läk. Tidn.* 1960, 57 3159–3173.

Hanson, L. The disruption of conditioned avoidance response following selective depletion of brain catechol amines. *Psychopharmacologia* (Berl) 1965, 8, 100.

Hanson, L. Biochemical and behavioural effects of tyrosine hydroxylase inhibition. *Psychopharmacologia* (Berl.) 1967 11 8–17

Hook, J B & K. E. Moore: The renal handling of α-methyltyrosine. *J Pharmacol. exp Ther* 1969 168 310–314.

Jönsson, L. & S. Ruberth. Ethylene glycol poisoning in dogs and cats. *Nord. Vet Med.* 1967 19 265–276

Magnusson, G H. Corrodi & E. Hansson Renal lesions induced by 3, α-dimethyltyrosine methylester-HCl. *Proc European Society Study Drug Toxicity* Xth Meeting, Oxford, April 1968, 206–217

Maitre, L. Presence of α-methyl-dopa metabolites in heart and brain of guinea pigs treated with α-methyl-tyrosine. *Life Sciences* 1965, 4, 2249

Moore, K. E., P F Wright & J K. Bert: Toxicological studies with α-methyltyrosine, an inhibitor of tyrosine hydroxylase. *J Pharmacol. exp Ther* 1967 155, 506–515.

Roberts, J A. & H. R. Seibold. Ethylene glycol toxicity in the monkey *Toxicol. appl. Pharmacol.* 1969 15 624–631.

Smith, H. A. & T C. Jones. *Veterinary pathology* Lea & Febiger Philadelphia, Pa., 1961 p. 626

Spector S., A. Sjoerdsma & S. Udenfriend. Blockade of endogenous norepinephrine synthesis by α-methyl-tyrosine, an inhibitor of tyrosine hydroxylase. *J Pharmacol. exp Ther* 1965 147 86

Weissman, A. & B. K. Koe: Behavioral effects of L-α-methyltyrosine, an inhibitor of tyrosine hydroxylase. *Life Sciences* 1965 4, 10.

Acta pharmacol. et toxicol. 1970 **28** 445–453

From the Research and Development Laboratories, Astra Pharmaceuticals, Södertälje, Sweden

Stereospecificity of the Enzymatic Biotransformation of the Enantiomers of Prilocaine (Citanest®)

By

B. Åkerman and S. Ross

(Received March 19 1970)

Abstract: No differences have been found between D-(−)- L-(+)- and DL-(±)-prilocaine (citanest®) with regard to intravenous LD50's in mice, nor have any differences been found between the D-(−)- and L-(+)-forms on subcutaneous injection. Compared to L-(+)-prilocaine, D-(−)-prilocaine was less toxic on intravenous infusion (mice, 0.12–0.50 mg/min.) and had less cumulative effect in rabbits (20 mg/kg each 15 min.). The plasma concentrations (cats) of the D-(−)-form (25 mg/kg intravenously) were lower than those of the L-(+)-form and more *o*-toluidine, one of the metabolites of prilocaine, was detected in the blood. D-(−)-prilocaine also produced methaemoglobinaemia at a more rapid rate. The ^{14}C-labelled enantiomers of prilocaine were hydrolyzed at significantly different rates by liver homogenates, slices (mice, rabbits, cats) and isolated microsomes (rabbits). D-(−)-prilocaine had the highest affinity for the enzyme(s). Thus, a significant stereospecificity of the hydrolyzing enzymes in their reaction with the enantiomers of prilocaine was found. However no evidence was obtained which could suggest substitution of the local anaesthetic prilocaine by either of its optical isomers.

Key-words: Anaesthetics, local – metabolism optical isomers prilocaine.

Recently there has been a growing interest in the relative effects of the enantiomers of local anaesthetic drugs. Evidence has been produced, showing that the enantiomers of aminoacylanilides may diverge significantly with regard to their local anaesthetic properties (Akerman *et al.* 1967 Luduena 1969 Adler *et al.* 1969). In addition to having practical consequences, such findings may also throw some light on the complexity of factors determining local anaesthetic potency. The results of studies on enantiomers of spirosuccinimides (Akerman *et al.* 1969 Akerman & Sokoll 1969) are even more interesting from a theoretical point of view since they indicate some degree of sensitivity to steric factors in the actual blocking mechanism. As far as

we know there are no reports on the biotransformation of optical isomers of local anaesthetics. We therefore decided to study the acute toxicity and metabolism of the D-(−)- and L-(+)-forms of prilocaine (α-propylamino-2 methylpropionanilide, citanest®) as compared to DL-(±)-prilocaine.

Material and Methods

The intravenous and subcutaneous LD50 values in male albino mice (18–22 g) were determined by injecting 0.2 % and 2.0 % solutions (saline pH 6.9) into the tail vein and into the back respectively. The method of MILLER & TAINTER (1944–45) was employed in calculating the LD50 values. Groups of 10 animals were used for each dose. The concentrations and doses in this and following experiments refer to the hydrochloride.

The intravenous toxicity at different rates of infusion was also studied in mice. 0.5 % solutions (pH 6.9) were given into the tail vein via a plastic catheter connected to 10 ml syringe driven by a Braun constant rate infusion pump.

In a third series of experiments, rabbits were given injections lasting 30 sec. into the marginal ear vein every 15 min. (ASTRÖM & PERSSON 1961). Each animal received 9 consecutive injections of 20 mg/kg in the form of 2.0 % solutions (pH 6.7 n = 6). The duration of the loss of the righting reflex was taken as a measure of the toxicity.

The methaemoglobin formation and the plasma concentrations of the compounds were studied following intravenous injections into healthy cats of both sexes (2.5–3.5 kg). The cats were anaesthetized with pentobarbitone sodium (mebumalum NFN), 30 mg/kg intraperitoneally. In the methaemoglobin studies 2.0 % solutions (pH 6.8–7.2) were given at a rate of 0.1 ml/min. into the femoral vein by means of Palmer slow injection apparatus. Blood samples were withdrawn at regular intervals up to 6 hours after commencement of infusion. The cats were given 20 mg/kg of the compounds and the blood samples (1.5 ml) were analyzed spectrophotometrically for methaemoglobin according to the method of EVELYN & MALLOY (1938). For the analysis of the plasma concentrations, the cats were given 25 mg/kg of the enantiomers. After centrifugation of the blood, the amounts of the compounds in plasma aliqots were determined by gas chromatographic analysis (SVINHUFVUD et al. 1965). The amount of o-toluidine formed was estimated in two cats.

^{14}C-Prilocaine labelled in the carbonyl carbon atom and resolved into the two enantiomers (TÉGNER & WILLMAN 1961, TÉGNER & DOMEIJ 1962) was used for the studies of the biotransformation. The enzymatic degradation of the compounds was determined by extracting the homogenized and alkalized tissue with n-heptane, and recording the radioactivity in the heptane solution by means of a liquid scintillation system (Packard Tri Carb) using a solution of 0.4 % 3,4-diphenyloxazole (PPO) and 0.01 % β-bis[2-(phenyloxazolyl)]-benzene (POPOP) in toluene as scintillation fluid. This procedure results in the extraction of prilocaine into the heptane, but the ^{14}C-labelled amino acid (N-propylalanine) formed by the hydrolyzing enzymes remains in the aqueous solution (AKERMAN et al. 1966a).

The hydrolysis of the enantiomers and the racemate by liver homogenate and slices was determined as described previously for prilocaine (AKERMAN et al. 1966a). The liver was homogenized in 10 volumes (w/v) of 0.05 N Tris buffer pH 8.5. The incubation mixture consisted of 0.5 ml of the homogenate and 0.4 µmoles of ^{14}C-labelled

compound in 0.5 ml distilled water. With regard to the slices, 250 mg in 3 ml of Krebs-Henseleit buffer (pH 7.4) containing 0.2 % glucose were incubated with 0.4 µmoles of the ^{14}C-labelled agents. The incubations were performed in a metabolic shaker at 37°. The reaction was arrested by the addition of 1.0 ml of 10 % trichloracetic acid (TCA) and the slices were homogenized in the incubation medium. The alkalinized homogenate was extracted with 20 ml of heptane and the radioactivity measured as described above.

Microsomes of rabbit liver were isolated by differential centrifugation. The homogenate contained in 10 volumes of 0.25 M sucrose was centrifuged at 25000 × g for 20 min. The supernatant liquid was then centrifuged at 105000 × g for 90 min. The pellet was resuspended in 0.25 M sucrose. Determination of the enzymatic hydrolysis of ^{14}C-prilocaine and its optical isomers at pH 8.5 (Tris buffer) by the microsome fraction was performed as described above for the homogenate.

Results

The intravenous LD50 values (mice) were: DL-(±)-prilocaine 37 ± 1.3 mg/kg, D-(−)-prilocaine 39 ± 1.3 mg/kg and L-(+)-prilocaine 40 ± 1.7 mg/kg. The subcutaneous values were 360 ± 14 and 380 ± 24 mg/kg for the D-(−)- and L-(+)-forms respectively. Thus the enantiomers and racemate of prilocaine did not differ significantly regarding the intravenous LD50 s nor did D-(−)-prilocaine differ from L-(+)-prilocaine on subcutaneous administration. As indicated in fig. 1 the duration of a toxic sign (loss of the righting reflex) increased on repeated intravenous injections of

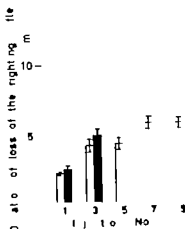


Fig. 1. Cumulative toxicity in rabbits. Durations (= 6) of loss of righting reflex following repeated slow intravenous injections (20 mg/kg) each of 15 min. interval. Open bars = D-(−)-prilocaine and black bars = L-(+)-prilocaine.

Table 1

Mice. Lethal doses determined for the enantiomers of prilocaine at different rates of intravenous infusion. 0.2 % solutions. n = 10 N S. = not significant

Compound	Lethal dose, mg/kg Infusion rate, mg/min. 0.12	P*	0.30	P	0.72	P
D − (−)-prilocaine	357 ± 20		211 ± 22		142 ± 11	
		0.01 > P > 0.001		0.05 > P > 0.01		N.S.
L − (+)-prilocaine	252 ± 13		144 ± 5		119 ± 4	

Significance of differences according to Wilcoxon rank test (SNEDECOR & COCHRAN 1967).

both enantiomers However the cumulative effect of L-(+)-prilocaine was more pronounced than that of the D-(−)-form. The intravenous toxicity of the enantiomers was also studied using constant rates of infusion. Table 1 shows that the D-(−)-form became significantly less toxic than L-(+)-prilocaine on decreasing the rate of infusion.

The plasma concentrations of D-(−)-prilocaine were lower than those of L-(+)-prilocaine after intravenous infusion into cats (fig. 2) The

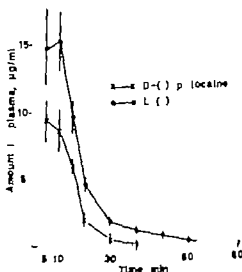


Fig. 2. Plasma concentrations in cats. The enantiomers (25 mg/kg, 2.0 % solution) were administered intravenously over a period of 15 min. (n = 4).

amounts of both enantiomers detected in the plasma decreased rapidly after termination of the infusion. D-(−)-prilocaine could not be detected in the 50 min. samples. The corresponding time for the L-(+)-form was about 80 min. Previous studies (AKERMAN *et al.* 1966a) have indicated that prilocaine is rapidly metabolized and split enzymatically at the amide bond. On analyzing the plasma samples from two of the cats, the amount of *o*-toluidine rose to 9.9 µg/ml plasma (15 min. samples) following treatment with D-(−)-prilocaine The corresponding peak value following treatment with the L-(+)-form was 7 0 µg/ml (15 min samples) No *o*-toluidine was detected in the 90 min and 120 min. samples, respectively

Evidence has been produced that the methaemoglobinaemia observed after high doses of racemic prilocaine (FUJIMORI *et al.* 1964 HJELM & HOLMDAHL 1965) is associated with the formation of metabolites subsequent to hydrolysis of the amide bond (AKERMAN *et al* 1966b) As shown in fig. 3 both D-(−)- and L-(+)-prilocaine produced methaemoglobin in cats.

The rate of methaemoglobin formation following injection of the D-(−)-form was significantly more rapid than with the L-(+)-form. Peak levels were thus reached later after injecting L-(+)-prilocaine (after about 3 hrs) as compared to the D-(−)-form (about 1 hr) but there was no statistically significant difference in the maximal level of methaemoglobinaemia. The rate of rise of methaemoglobin level in the blood observed with the racemate was between that of the two enantiomers.

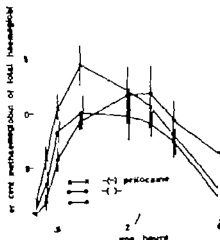


Fig. 3 Formation of methaemoglobin in cats, expressed as percentage of the haemoglobin. 20 mg/kg of D-(−)-prilocaine (= 8), L-(+)-prilocaine (n = DL-(±)-prilocaine (= 7) were given in 2.0 % solutions t a rate of 0.1

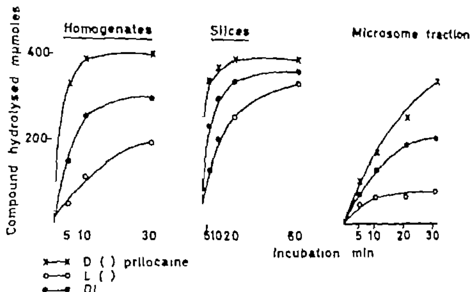


Fig. 4. Enzymatic hydrolysis of ^{14}C-labelled prilocaine and the enantiomers by homogenates, slices and microsome fraction of rabbit liver. The concentration of compound in the incubation mixture was 400 μmoles per ml for the homogenate and the microsome fraction and for slices 133 μmoles per ml. The amount of liver tissue was 50 mg and 250 mg for the homogenates and slices respectively. The microsomes were prepared from 50 mg liver tissue.

The findings of the *in vivo* studies indicated a difference in the rate of metabolism of the enantiomers of prilocaine. Subsequent *in vitro* experiments demonstrated that D-(−)- and L-(+)-prilocaine were hydrolysed by homogenates, slices and isolated microsomes of liver at significantly different rates (fig. 4). The rate of hydrolysis increased in the order L-(+)-form, racemate and D-(−)-form. Liver from rabbits was more active than liver from cats and mice. The soluble fraction after high speed centrifugation of liver homogenate was without any hydrolyzing activity. The enzymes responsible for the hydrolysis of prilocaine and the enantiomers were thus located exclusively in the microsome fraction and showed a high selectivity for the D-(−)-form. The microsomes failed to hydrolyse more than about 20 per cent of the amount of the L-(+)-enantiomer present in the incubation mixture. The pH-optimum of the reaction was about 8.5–9.0 (fig. 5). SKF 525 A (10^{-3}M) inhibited the enzymatic hydrolysis of L-(+)-prilocaine by 53 per cent after an incubation time of 20 min. The corresponding inhibition of the reaction with D-(−)-prilocaine was 22 per cent. The L-(+)-form was thus affected more than the D-(−)-form.

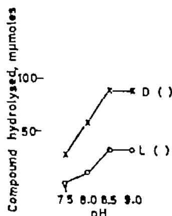


Fig. 5. pH dependence of the enzymatic hydrolysis of D-(−)- and L-(+)-prilocaine by the microsome fraction of rabbit liver. The incubation time was 5 min.

Discussion

In this study the enantiomers and the racemic form of the local anaesthetic prilocaine (citanest®) were found to have similar intravenous LD50 values and they did not differ in toxicity on rapid intravenous infusion into mice. A close correlation between toxicity expressed as intravenous LD50 values, and activity at excitation block *in vitro* has been demonstrated for compounds in the series of ammoacylanilides to which prilocaine belongs (AKERMAN *et al.* 1966a, AKERMAN *et al.* unpublished). The enantiomers of prilocaine have the same blocking effect on isolated nerves (AKERMAN *et al.* 1967). It is therefore likely that the lack of difference between the enantiomers and the racemate in the above mentioned tests is mainly related to an identical intrinsic toxic action on the central nervous system. On the other hand D-(−)-prilocaine had a lower cumulative toxicity than the L-(+)-form and became less toxic at slower intravenous infusion rates. Furthermore D-(−)-prilocaine reached lower plasma concentrations and produced methaemoglobinaemia at a more rapid rate than L-(+)-prilocaine. These differences between the enantiomers may be explained by unequal rates of biotransformation.

The metabolism of prilocaine *in vivo* seems to result chiefly in hydrolysis at the amide linkage (GEDDES 1965, AKERMAN *et al.* 1966a). The enzyme activity of the liver which may be mainly responsible for the hydrolysis *in vivo* was located exclusively in the microsome fraction. This had the same pH-dependency as that found by HOLLUNGER (1960) in the hydrolysis of monoethylglycine xylidide and thus these two fractions are probably identical. One or more enzymes may be involved; no attempt has been made to elucidate this problem. The significantly faster hydrolysis of D-(−)-prilo-

caine found *in vitro* is in accordance with the observation of a more rapid hydrolysis *in vivo*

Comparison of the local anaesthetic effect of L-(+)-prilocaine and D-(−)-prilocaine in various tests in animals indicates that the former has a blocking effect in lower concentrations and is more long-acting on injection of solutions of equal strength (AKERMAN *et al* 1967) Evidence was produced that this difference is related to a better localization of the L-(+)-form at the site of application. A similar explanation has been proposed for the longer durations of (−)-bupivacaine and (+)-mepivacaine as compared to their enantiomers (LUDUENA 1969). Since D-(−)-prilocaine was metabolized more rapidly one would have expected it to be less toxic than L-(+)-prilocaine on subcutaneous administration. However the enantiomers did not differ significantly in this respect. This may be because the differences in the rates of metabolism are too small to be evident in this type of test as well as on rapid intravenous injection. A contributing factor may also be that the effect of a somewhat more rapid breakdown of D-(−)-prilocaine is compensated by this form being more quickly absorbed than the L-(+)-form according to the investigations mentioned above Since the enantiomers of prilocaine, like the racemic form, appear to be metabolized mainly in the liver It does not seem particularly credible that, for example the somewhat better local anaesthetic effect of L-(+)-prilocaine on local application can be related to a somewhat slower rate of destruction as compared to the D-(−)-form.

Even if the L-(+)-form of prilocaine has proved to be a slightly more potent local anaesthetic than the D-(−)-form the differences as compared with the racemate are not significant (unpublished results) These findings and the lack of any real differences in toxicity and methaemoglobin formation between either of the enantiomers and the racemate indicate that there would be no advantage in substituting prilocaine (citanest®) by the L-(+)-form or still less by the D-(−)-form.

Stereospecificity has not been found for the pairs of enantiomers referred to above in their reaction with sites in the excitable membrane associated with block. Instead the differences in the local anaesthetic effect could be related to mechanisms other than the block of action potential generation. However findings with enantiomers of other types of compound indicate that steric interaction of the drug with the site of action is of some significance in the excitation block (AKERMAN *et al.* 1967 CAMOUGIS *et al.* 1967 SCHÖNTENBERGER *et al.* 1967 AKERMAN *et al* 1969). Steric factors may thus influence local anaesthesia both at the site of action and by indirect mechanisms. In order to explain any difference that might be found in the local anaesthetic effect between enantiomers, one has therefore to study more than one biological system. One more reason for this is that the effects of enantiomers in different systems will very likely vary with the type of compound used.

REFERENCES

Adler R., G Adler & G Aberg: Effects of optically active isomers and racemat of meplvacaine (carbocaine®) in dental anesthesia. *Svensk Tandlakare Tidskrift* 1969 8, 501 504.

Akerman, B., A. Aström, S. Ross & A. Telc Studies on the absorption, distribution and metabolism of labelled prilocaine and lidocaine in some animals species. *Acta pharmacol. et toxicol.* 1966a, 24 389–403.

Akerman, B S. A. Pettersson & P Wistrand Methemoglobin forming metabolites of prilocaine. *III International Pharmacological Congress Sao Paul Brazil* 1966b, Abstracts, 237

Akerman, B., N H. Persson & C. Tegnér Local anaesthetic properties of the optically active isomers of prilocaine (citanest®) *Act pharmacol et toxicol.* 1967 25 233–41

Akerman, S. B. A G Camougis & R. A. Sandberg Stereoisomerism and differential activity in excitation block by local anesthetics. *Europ J Pharmacol.* 1969 8, 337 347

Akerman, B. & M. Sokoll. The effect of a pair of enantiomers with local anesthetic activity on action potential generation and acetylcholine sensitivity in muscle. *Europ J Pharmacol.* 1969 8, 331 336.

Aström, A. & N. H. Persson. Some pharmacological properties of α-methyl-n-propylaminopropionanilide, new local anesthetic. *Brit J Pharmacol. Chemother* 1961 16, (1), 32–44

Camougis, G B. Akerman & R. Sandberg Stereoisomerism and differential potency of excitation block. *Pharmacologist* 1967 9 205

Evelyn, K. A. & H. T Malloy Microdetermination of oxyhemoglobin, methemoglobin and sulfhemoglobin in single sample of blood. *J Biol Chem.* 1938, 126, 655–662.

Fujimori, M., K. Nishimura & E. Goto Methemoglobinemia due to local anesthetics (a preliminary report). *Far East J Anesthesia* 1964 4 22 30.

Geddes, I. C.. Studies of the metabolism of Citanest C14 *Acta Anaesth Scand.* 1965, *Suppl. XVI* 37–44

Hjelm, M. & M. H-von Holmdahl. Clinical chemistry of prilocaine and clinical evaluation of methaemoglobinaemia induced by this agent. *Acta Anaesth Scand.* 1965, *Suppl. XVI* 161 170

Hollunger G Some characteristics of an amide-hydrolysing microsomal enzyme before and after its solubilization *Acta pharmacol. et toxicol.* 1960, 17 384–389

Lüduena, F P Duration of local anesthesia. *Ann. Rev Pharmacol.* 1969 9 503–520.

Miller L. C. & M. L. Tainter Estimation of the ED50 and its error by means of logarithmic-probit graph paper *Proc Soc. Exp Biol. Med* 1944–45, 57 58, 261 264

Schoenenberger H., K. D Pochsberger A. Petter & R. Brinkmann. Wechselbeziehungen zwischen Wirkstoff und Rezeptor bei lokalanästhetisch wirksamen N-Aminoacylephedrinen. *Pharm. Acta H I* 1967 42, 163–176.

Snedecor G W & W C. Cochran. *Statistical methods.* Ames, Iowa, 1967 6. edition.

Svinhufvud, G., B. Örtengren & S. E. Jacobsson. The estimation of lidocaine and prilocaine in biological material by gas chromatography *Scand. J Clin. Lab Invest* 1965, 17 162–164.

Tegnér C. & N H Willman. Resolution of α-n-propylamino-2-methyl-propionanilide and the absolute configuration of its stereoisomers. *Acta Chem. Scand.* 1961 1180–1181

Tegnér C. & K. E. Domeij Syntheses of α-propylamino-2-methyl-(carbonyl-)propionanilide. *Acta. Chem. Scand.* 1962, 16, 1041–1042.

Acta pharmacol et toxicol 1970, 28, 454–465

From the Department of Pharmacology Institute of Pharmacy
University of Oslo, Norway

Effects of Tetraethylthiuram Disulphide (Disulfiram), Diethyldithiocarbamate and Ethanol on Factors of the Kinin System in Human Blood

By

Anne Marie Vesterød

(Received April 16, 1970)

Abstract Tetraethylthiuram disulphide (disulfiram) and diethyldithiocarbamate were tested for kininase-inhibiting effect (*in vitro* experiments). Disulfiram was found to be a potent inhibitor of human erythrocyte kininase (inhibition obtained at 25 μg/ml incubation mixture), but it did not inhibit human plasma kininase (in concentrations up to 200 μg/ml) Diethyldithiocarbamate was a potent inhibitor of plasma kininase (inhibition obtained at 25–50 μg/ml), but had a weaker effect on erythrocyte kininase (100–200 μg/ml being required for inhibition). Ethanol in the concentration range 5–10 % (w/v) was found to release kinin in human citrated plasma stabilized with EDTA 2Na, 4 mg per ml. I minor concentrations (0.5–4 % (w/v) were tested) ethanol potentiated the kinin-releasing effect of ellagic acid. Comparative determinations were made of kininase, prekallikrein, total kininogen, kininogen fractions, and the rate of release of kinin in the plasma from 3 males on disulfiram-treatment (daily dose 0.2 g) and also from untreated males. The results did not show any differences between the two groups.

Key-words: Disulfiram – ethanol – kinin – kininase.

In 1948, HALD *et al* introduced disulfiram in the treatment of chronic alcoholism, based on the observation that moderate doses of ethanol to humans pretreated with disulfiram elicited various unpleasant symptoms (disulfiram-ethanol reaction) Along with several other workers they shortly afterwards obtained evidence indicating that disulfiram *in vivo* inhibited the oxidation of acetaldehyde but left the oxidation of ethanol to acetaldehyde unaffected. The conclusion was that most of the symptoms of the disulfiram-ethanol reaction in man are caused by an accumulation of acetaldehyde in the organism (HALD & JACOBSEN 1948 ASMUSSEN *et al* 1948 LARSEN 1948 HALD *et al.* 1949 KJELDGAARD 1949 GRAHAM 1951).

ELDJARN (1950) and STRÖMME (1964) showed that disulfiram in the organism is readily reduced to diethyldithiocarbamate. ELDJARN suggested that

diethyldithiocarbamate is responsible for the effect on the metabolism of ethanol.

It is well known that the disulfiram-ethanol reaction in man is associated with a pronounced fall in blood pressure, while the effect of acetaldehyde *per se* is sympathomimetic. PERMAN (1962) suggested that the hypotensive effect of the disulfiram-ethanol reaction is primarily due to a blood-borne factor with peripheral action and not to acetaldehyde *per se* and RABY (1956) suggested that the hypotension may be due, at least partially to some substance other than acetaldehyde. STRÖMME (1964) on the other hand, put forward the theory that an acute insufficiency of the glucuronic acid-conjugation system might be the cause of the disulfiram-ethanol reaction. He demonstrated that injection of disulfiram and ethanol interfered with this system, and suggested that this might cause serious reactions, either through an inhibition of the conjugation of endogenous compounds, or by way of a decreased detoxification of disulfiram itself.

In the present work disulfiram and diethyldithiocarbamate have been tested for kininase-inhibiting effect (*in vitro* experiments). Ethanol has been tested for kinin-releasing effect, both alone and in combination with plasma kallikrein or ellagic acid (*in vitro* experiments) Different factors of the plasma kinin system were determined in the blood from males treated with disulfiram and in the blood from healthy untreated males.

Technique

A. *Materials and assay*

Plasma specimens from 3 disulfiram-treated males (a daily dose of 0.2 g for at least one week) and batches of the pooled plasma from at least 3 healthy untreated males were used for the preparation of plasma substrate, plasma kallikrein, and plasma kininase preparations, while the erythrocyte kininase preparations were made from blood samples from healthy males.

1. *Plasma substrate* Human blood was collected by venepuncture into siliconized, graduated cylinder containing 1 ml of 3.1 % sodium citrat dihydrate solution per 9 ml of blood, transferred to siliconized centrifuge tube, and centrifuged at 1.3 × 10^3 g for 30 minutes at 10° 4 mg EDTA 2N dissolved in 0.04 ml of water was then added per ml of citrated plasma with subsequent heating for 30 minutes t 37° The substrate was stored at −20 1 ml corresponds to 0.81 ml plasma (BRISEID *et al* 1967).

2. *Plasma kallikrein.* Citrated plasma was prepared as described bove for the plasma substrate (EDTA-treatment omitted) and stored at −20 For kallikrein activation, 0.20 ml acetone was added per ml citrated plasma (16.7 % (v/v) of acetone), and the mixture was left for about 17 hours at room temperature (22 ± 2°). The activity was llminated by incubation with EDTA as stated bove, and the kin preparation was then ready for assay (BRISEID *et al.* 1968b).

3 *Plasma kininase.* Citrated plasma (prepared as described above, but the EDTA treatment omitted) was shaken for 30 minutes with 50 mg of silica powder per ml of citrated plasma. The silica was removed by centrifugation and decantation and the kininase preparation was stored at −20 (RINVIK *et al.* 1966).

4 *Erythrocyt kininase.* The erythrocyte fraction from one blood sample was washed by means of a 4-times repeated suspending on an equal volume of 0.9 % sodium chloride solution and centrifugation for 15 minutes at 1.3 × 10³ g. After the last centrifugation and decantation, 1 ml samples were pipetted off and stored at −20° (RINVIK *et al.* 1966).

5 *Ellagic acid solution.* 1.10⁻⁴M in saline with sodium bicarbonate added to pH 7.5.

6. *Reagents. Bradykinin* Sandoz, AG Basel, Switzerland. *Disulfiram* antabus® Dumex Chemical Division, Dumex Limited, Copenhagen, Denmark. *Sodium diethyldithiocarbamate* Eastman Organic Chemicals, Rochester 3, New York, U.S.A. *Padutin*® (Hog pancreas kallikrein), Bayer AG Leverkusen, Germany *Ellagic acid*, Practical grade, Sigma Chemical Company St. Louis, Missouri, U.S.A.

7 *Assays.* The kinin determinations were generally carried out on the isolated rat uterus as "bracketing assays with a dose ratio of 3 2. Bradykinin was used as standard substance.

B *Methods.*

1. *Determination of the kininase-inhibiting effects of disulfiram and diethyldithiocarbamate*

From preliminary experiments, the concentration of enzyme required to break down about 70–90 % of a standard concentration of bradykinin (1.25 μg/ml) in a standard time (8 minutes) was estimated. Next, an inhibitor-effect curve was established by adding different amounts of inhibitor to the enzyme and substrate concentrations chosen, the other experimental conditions remaining unchanged. The concentration of inhibitor found to inhibit partly the inactivation, 20–50 % of bradykinin being broken down after 8 minutes, was then used in a final experiment in which the inactivation was recorded at 8, 16, and 32 minutes respectively

The experiments were carried out in tris buffer (0.1 M, pH 7.5); disulfiram was added dissolved in acetone, 0.05 ml per 1.0 ml incubation mixture, the other chemicals and the kininase preparations in 0.9 % sodium chloride solution. Acetone was also added to the control solutions incubated in parallel, since acetone alone inhibits the kininases. Incubations were carried out at 37

The inactivation experiments were stopped by adding 0.04 N hydrochloric acid to a pH of about 1.8, 3.0 ml acid to 1.0 ml incubation mixture being needed, and then heating for 10 minutes at 37 Just before the assay 0.1 N sodium hydroxide was added to pH 7.5 ± 0.1, about 1.45 ml being required (DYRUD *et al.* 1965).

2. *Estimation of the kinin-releasing effect of ethanol.*

The kinin-releasing effect of ethanol in human plasma substrates was examined for the concentration range 2.5–20.0 % (w/v), while the influence of ethanol on the kinin release caused by human plasma kallikrein or by ellagic acid was examined for the concentration range 0.5–4.0 % (w/v). The incubations were carried out at 37° and aliquots were diluted with saline, heated for 5 minutes at 100 and assayed on the isolated rat uterus. Further experimental details are given in tables 3 and 4

3. *Determination of different factors of th kinin system in human plasma.*

Plasma kininase To various amounts of the plasma kininase preparation (usually 0.15 0.20, 0.30, 0.40 ml) were added 1.25 μg bradykinin in 0.125 ml of 0.9 % sodium chloride solution and tris buffer (0.1 M, pH 7.5) to 1.00 ml. The mixture was incubated for exactly 8 minutes at 37 The further procedure was as described bove for the kininase inhibitor experiments.

A linear log-concentration response curve was drawn and the plasma kininase activity was given as th amount f citrated plasma in ml per ml incubation mixture that inactivated 50 % f the substrate under the conditions described (RINVIK *et al.* 1966).

Determination of the total kininogen by the acetone activation method. To 1.0 ml plasma substrate were added 3.5 ml 0.9 % sodium chloride solution and 0.5 ml acetone. After 5-minute equilibration period in a waterbath t 37 the tube was stoppered and the incubation continued for 24 hours. A 0.5 ml sample was withdrawn, 2.5 ml of saline added, and the mixture heated for 5 minutes at 100 in a waterbath (BRISEID *et al.* 1967).

Determination of the rat and extent f kinin release caused by padutl To three 1.0 ml samples (a, b,) of plasma substrat were added 0.35 0.70, and 4.00 units f padutin respectively and saline up to 5.00 ml and the mixture was then incubated at 37° After 7.5, 15 30, and 60 minutes, 0.5 ml samples were withdrawn from tubes and b and the reaction stopped by heating for 5 minutes in boiling waterbath after dilution with 2.5 ml of boiling saline. From tube 0.5 ml sampl was withdrawn after only 60 minutes. The kinin values were calculated as μg bradykinin per ml plasma, and the amounts obtained after 7.5, 15, and 30 minutes incubation for both submaximal kallikrein concentrations (0.35 and 0.70 units) were converted to percentages of the amounts obtained after 60 minutes incubation with the same enzyme concentrations. Log-minute release curves were drawn and the time intervals corresponding to 66 % release were estimated (BRISEID *et al.* 1967).

Determination f the extent of kinin release caused by human plasma kallikrein. To 1.0 ml sample of plasma substrate was added 1.0 ml plasma kallikrein and the mixtur was incubated t 37 for 10 minutes. A 0.2 ml aliquot was withdrawn and the reaction stopped by heating for 5 minutes in boiling waterbath after d lution with 2.5 ml of boiling saline.

Determination f plasma prekallikrein. T each of four 1.0 ml samples of plasma substrate, were added 0.14 ml of cetone and then 0.04, 0.06, 0.10, and 0.15 ml of kallikrein preparation respectively. T one sample was added 0.09 ml acetone and 1.0 ml of kallikrein preparation were added. A blind test with 0.14 ml of acetone, but without any enzyme added, was used as control. After 10 minutes incubation at 37° 0.2 ml samples were withdrawn and the reaction stopped by heating for 5 minutes in boiling waterbath after dilution with 2.5 ml of boiling saline. The kinin values were calculated as μg bradykinin/ml plasma, and the amounts obtained with the 4 submaximum kallikrein concentrations were converted to percentages of the amount obtained with excess of enzyme preparation, i.e. 1 ml per ml substrate. A concentration-effect curve was drawn and the amount of kallikrein corresponding to 50 % was estimated (BRISEID *et al.* 1968b).

The enzyme preparations from the disulfiram-treated males were assayed in with the enzyme preparations from healthy untreated males.

3. *Plasma kininase.* Citrated plasma (prepared as described above, but the EDTA-treatment omitted) was shaken for 30 minutes with 50 mg of silica powder per ml of citrated plasma. The silica was removed by centrifugation and decantation and the kininase preparation was stored at −20 (RINVIK *et al.* 1966)

4. *Erythrocyte kininase.* The erythrocyte fraction from one blood sample was washed by means of a 4-times repeated suspending on an equal volume of 0.9 % sodium chloride solution and centrifugation for 15 minutes at 1.3×10^3 g. After the last centrifugation and decantation, 1 ml samples were pipetted off and stored at −20° (RINVIK *et al.* 1966)

5. *Ellagic acid solution* 1 10^{-4}M in saline with sodium bicarbonate added to pH 7.5.

6. *Reagents Bradykinin* Sandoz, AG Basel, Switzerland. *Disulfiram*, antabus® Dumex Chemical Division, Dumex Limited, Copenhagen, Denmark. *Sodium diethyldithiocarbamat* Eastman Organic Chemicals, Rochester 3, New York, U.S.A. *Padutin®* (Hog pancreas kallikrein), Bayer AG Leverkusen, Germany *Ellagic acid*, Practical grade, Sigma Chemical Company St. Louis, Missouri, U.S.A.

7. *Assays.* The kinin determinations were generally carried out on the isolated rat uterus as "bracketing assays with a dose ratio of 3 2. Bradykinin was used as standard substance.

B. *Methods.*

1. *Determinatio of the kininase-inhibiting effects of disulfiram and diethyldithiocarbamat*

From preliminary experiments, the concentration of enzyme required to break down about 70–90 % of a standard concentration of bradykinin (1.25 µg/ml) in a standard time (8 minutes) was estimated. Next, an inhibitor-effect curve was established by adding different amounts of inhibitor to the enzyme and substrate concentration chosen, the other experimental conditions remaining unchanged. The concentration of inhibitor found to inhibit partly the inactivation, 20–50 % of bradykinin being broken down after 8 minutes, was then used in a final experiment in which the inactivation was recorded at 8, 16, and 32 minutes respectively

The experiments were carried out in tris buffer (0.1 M pH 7.5), disulfiram was added dissolved in acetone 0.05 ml per 1.0 ml incubation mixture, the other chemicals and the kininase preparations in 0.9 % sodium chloride solution. Acetone was also added to the control solutions incubated in parallel, since acetone alone inhibits the kininases. Incubations were carried out at 37

The inactivation experiments were stopped by adding 0.04 N hydrochloric acid to a pH of about 1.8, 3.0 ml acid to 1.0 ml incubation mixture being needed, and then heating for 10 minutes at 37 Just before the assay 0.1 N sodium hydroxide was added to pH 7.3 ± 0.1, about 1.45 ml being required (DYRUD *et al.* 1965).

2. *Estimation of th kinin-releasing effect of ethanol.*

The kinin-releasing effect of ethanol in human plasma substrates was examined for the concentration range 2.5–20.0 % (w/v), while the influence of ethanol on the kinin release caused by human plasma kallikrein or by ellagic acid was examined for the concentration range 0.5–4.0 % (w/v). The incubations were carried out at 37 and aliquots were diluted with saline, heated for 5 minutes at 100 and assayed on the isolated rat uterus. Further experimental details are given in tables 3 and 4.

3. *Determination of different factors of the kinin system in human plasma.*

Plasma kininase. To various amounts of the plasma kininase preparation (usually 0.15, 0.20, 0.30, 0.40 ml) were added 1.25 μg bradykinin in 0.125 ml of 0.9 % sodium chloride solution and tris buffer (0.1 M, pH 7.3) to 1.00 ml. The mixture was incubated for exactly 8 minutes at 37°. The further procedure was as described above for the kininase inhibitor experiments.

A linear log-concentration response curve was drawn and the plasma kininase activity was given as the amount of citrated plasma in ml per ml incubation mixture that inactivated 50 % of the substrate under the conditions described (RINVIK et al. 1966).

Determination of the total kininogen by the acetone activation method. To 1.0 ml plasma substrate were added 3.5 ml 0.9 % sodium chloride solution and 0.5 ml acetone. After a 5-minute equilibration period in a waterbath at 37° the tube was stoppered and the incubation continued for 24 hours. A 0.5 ml sample was withdrawn, 2.5 ml of saline added, and the mixture heated for 5 minutes at 100° in a waterbath (BRISEID et al. 1967).

Determination of the rate and extent of kinin release caused by padutin. To three 1.0 ml samples (a, b, c) of plasma substrate were added 0.35, 0.70, and 4.00 units of padutin respectively and saline up to 5.00 ml and the mixture was then incubated at 37°. After 7.5, 15, 30, and 60 minutes, 0.5 ml samples were withdrawn from tubes a and b and the reaction stopped by heating for 5 minutes in a boiling waterbath after dilution with 2.5 ml of boiling saline. From tube c a 0.5 ml sample was withdrawn after only 60 minutes. The kinin values were calculated as μg bradykinin per ml plasma, and the amounts obtained after 7.5, 15 and 30 minutes incubation for both submaximal kallikrein concentrations (0.35 and 0.70 units) were converted to percentages of the amounts obtained after 60 minutes incubation with the same enzyme concentrations. Log-minute release curves were drawn and the time intervals corresponding to 66 % release were estimated (BRISEID et al. 1967).

Determination of the extent of kinin release caused by human plasma kallikrein. To a 1.0 ml sample of plasma substrate was added 1.0 ml plasma kallikrein and the mixture was incubated at 37° for 10 minutes. A 0.2 ml aliquot was withdrawn and the reaction stopped by heating for 5 minutes in a boiling waterbath after dilution with 2.5 ml of boiling saline.

Determination of plasma prekallikrein. To each of four 1.0 ml samples of plasma substrate, were added 0.14 ml of acetone and then 0.04, 0.06, 0.10, and 0.15 ml of kallikrein preparation respectively. To one sample was added 0.08 ml acetone and 1.0 ml of kallikrein preparation were added. A blind test with 0.14 ml of acetone, but without any enzyme added, was used as control. After 10 minutes' incubation at 37° 0.2 ml samples were withdrawn and the reaction stopped by heating for 5 minutes in a boiling waterbath after dilution with 2.5 ml of boiling saline. The kinin values were calculated as μg bradykinin/ml plasma, and the amounts obtained with the 4 submaximum kallikrein concentrations were converted to percentages of the amount obtained with excess of enzyme preparation, i.e. 1 ml per ml substrate. A concentration-effect curve was drawn and the amount of kallikrein corresponding to 50 % release was estimated (BRISEID et al. 1968b).

The enzyme preparations from the disulfiram-treated males were assayed in parallel with the enzyme preparations from healthy untreated males.

Results

Disulfiram and diethyldithiocarbamate as inhibitors of human plasma kininase and erythrocyte kininase in vitro

Tables 1 and 2 show the results of the kininase inhibitor experiments. As will be seen from table 1 diethyldithiocarbamate sodium had a relatively weak inhibiting effect on human erythrocyte kininase. The concentration necessary for inhibition was rather high, and experiments with incubation periods of 16 and 32 minutes demonstrated that the inactivation of kinin was not stopped. In some experiments we preincubated the erythrocyte kininase with diethyldithiocarbamate for 60 minutes before adding the substrate to see if the poor effect might to some extent be attributed to the short standard incubation time used i. e. 8 minutes. DYRUD *et al.* (1965) found that the kininase-inhibiting effect of EDTA 2Na was considerably increased by such

Table 1

Inactivatio f bradykinin by human erythrocyte kininase in the presence of diethyldithiocarbamate sodium or disulfiram

The figures given refer to 1.0 ml incubation mixture with 1.25 μg bradykinin and 0.02–0.03 ml enzyme preparation. For further details see text.

Inhibitor	Time in minutes		Inhibitor	Bradykinin
	Preincub.	Incub.	μg	%
Diethyldithiocarbamate sodium	0	8	0	10
	–	–	100	12
	–	–	200	24
	–		350	40
	60	–	0	48
	–	–	50	13
	–		100	19
	–		200	30
	0	–	0	10
	–	–	350	40
	–	16	–	20
	–	32	–	6
Disulfiram	0	8	0	30
		–	25	71
	–	–	50	80
		–	100	83
		–	0	30
			50	80
		16	–	71
	–	32	–	54

Table 2

Inactivation of bradykinin by human plasma kininase in the presence of diethyldithiocarbamate or disulfiram.

The figures refer to 1.0 ml incubation mixture with 1.25 µg bradykinin and about 0.40 ml enzyme preparation. For further details see text.

Inhibitor	Incub time in minutes	Inhibitor µg	Brady-kinin %	Incub. time i minutes	Inhibitor µg	Brady kinin %
	8	0	22	8	0	23
Diethyldithio-	–	25	30	–	75	[illegible]
carbamate	–	50	40	16	–	7
sodium	–	100	64	32	–	0
Disulfiram	No inhibition at 200 µg					

a preincubation procedure. The effect of diethyldithiocarbamate, however was not increased after preincubation on the contrary the inactivation of bradykinin now seemed to be facilitated, especially at the lowest inhibitor concentrations (table 1) Disulfiram, on the other hand, was found to be a potent inhibitor of the erythrocyte kininase (table 1).

Table 2 shows that diethyldithiocarbamate is a relatively potent inhibitor of human plasma kininase, but that the inhibiting effect was short-lasting. Disulfiram had no inhibiting effect on the plasma kininase in concentrations up to about 200 µg/ml. Higher concentrations could not be tested because disulfiram was precipitated in the incubation mixture.

The influence of ethanol on the in vitro release of kinin in human plasma

Table 3A shows the results of a 22 hour experiment carried out with different ethanol concentrations. The ethanol concentration had to be rather high (about 5 0 to 7.5 / (w/v)), to give a significant release of kinin. [illegible] *et al.* (1968a) obtained similar results with acetone. In their experiments they also showed that 20 / (v/v) acetone released significantly less [illegible] than did 10 / (v/v) acetone, while the experiments [illegible] table 3A demonstrated the same kinin release with [illegible] / (w/v) [illegible] with 10 / (w/v) ethanol.

Table 3B shows the relation between the incubation time and [illegible] release at 10 % (w/v) ethanol. As will be seen the release was about 100 / after three hours. For acetone the [illegible] release [illegible] after about six hours incubation time ([illegible] et al. 19[illegible]).

Table 3

Significance f the ethanol concentration and of the incubation time for the release of kinin

Plasma substrate was diluted with mixtures of ethanol and saline to a final dilution of 1/1.2 (v/v)* and incubated at 37 The kinin released was calculated as µg bradykinin/ml plasma.

A. Incubation for 22 hours. Different ethanol concentrations.

B Incubation for varying periods of time. Ethanol concentration 10 % (w/v).

	Ethanol conc. % (w/v)	0	2.5	5.0	.5	100	20.0		
A	Substrate I	0.4	0.5	0.9	3 1	4.0	–		
	Substrate II	0.3	0.2	0.4	4 4	5.2	–		
	Substrate III	0.3	0.4	0.6	2.7	3.3	3 3		
B	Incub. time in hours	1	3	5	7	9	11	13	24
	Substrate III	0.3	3.1	3.5	3.3	3.3	3.3	3 3	3.4

(1/1.4 (v/v) when the ethanol concentration was 20 % (w/v))

Table 4A shows the results of 10-minute experiments in which human plasma substrate was incubated with both human plasma kallikrein and ethanol. The average amounts of kinin released by 20 µl enzyme and by 40 µl enzyme preparation per ml substrate were about 0.50 µg/ml and about 0 75 µg/ml respectively In control experiments without enzyme the average amount of kinin released was about 0 15 µg/ml substrate, and the same release value was obtained when 4 / (w/v) ethanol was present. The maximum release value obtained with an excess amount of plasma *kallikrein* was about 1 7 µg/ml substrate. The results shown in table 3A *might indicate* a slight potentation of the kinin-releasing effect of plasma kallikrein by the highest ethanol concentrations used, but the effect was not significant.

Table 4B shows the results of 60-minute experiments in which human plasma substrate was incubated with both ellagic acid and ethanol. The average amounts of kinin released by ellagic acid 2 10^{-6}M and 4 10^{-6}M were about 0 60 µg/ml and about 0.80 µg/ml respectively when no ethanol was present In control experiments without any ellagic acid the average amount of kinin released was about 0 14 µg/ml substrate and an insignificantly higher release value was obtained in the presence of 4 / (w/v) ethanol, 0.24 µg/ml substrate. As will be seen from the results in table 4B there is a significant potentation of the kinin releasing effect of ellagic acid by ethanol

Table 4

Influence / ethanol on the kinin-releasing effect of human plasma kallikrein and of ellagic acid.

A. Ethanol and plasma kallikrein. Plasma substrate was diluted with mixtures of ethanol and saline and then plasma kallikrein preparation to final dilution of 1/1.2 (v/v) and incubated for 10 minutes at 37°

B. Ethanol and ellagic acid. Plasma substrate was diluted with mixtures of ethanol and ellagic acid solution to final dilution of 1/2 (v/v) and incubated for 60 minutes at 37°

The kinin released was calculated as µg bradykinin/ml plasma and is given in the table as per cent of the kinin released by an excess amount of plasma kallikrein preparation, 1 ml per ml substrate. Average release value: 1 7 µg.

	Ethanol conc. % (w/v)	0	4.0	0	0.5	1.0	2.0	4.0	0	0.5	1.0	2.0	4.0
	Plasma kallikrein	–		20 µl					40 µl				
A	% kinin release, mean values	9	9	30	31	31	32	36	44	40	44	48	67
	Number of experiments	4	3	5	5	5	5	5	5	1	1	1	1
	Ellagic acid	–		$2\ 10^{-4}$M					$4\ 10^{-6}$M				
B	% kinin release, mean values	8	14	34	39	42	48	55	47	–	–	–	–
	Number of experiments	5	5	5	5	5	5	5	5	–	–	–	–

Table 5

Determination of plasma prekallikrein and f plasma kininase in plasma.

Kallikrein preparations (I) and kininase preparations (II) from respectively 3 and 6 males treated with disulfiram were prepared and tested separately (B), while the corresponding control preparations were made from the pooled plasma from 4 healthy untreated males (A). Test and control kallikrein preparations were determined in parallel. For further details see text.

	Kinin system parameter	A	B	B/A
I	µl kallikrein preparation per ml plasma substrate causing 50 % kinin release	123	100	0.8
		110	101	0.9
		78	81	1.0
	Mean	104	94	0.9
II	ml kininase preparation per ml test solution causing 50 % bradykinin inactivation	0.29	0.30	
			0.28	
			0.30	
			0.23	
			0.34	
			0.24	
	Mean		0.28	

Determination of different factors of the plasma kinin system in plasma from healthy untreated men and from men treated with disulfiram.

Tables 5 and 6 show the results of determinations of plasma prekallikrein, plasma kininase, total plasma kininogen, different fractions of plasma kininogen and the rate of release of kinin in plasma from untreated men and plasma from men treated with disulfiram. As will be seen from the tables the results give no evidence of significant differences in the above mentioned plasma kinin factors in these two groups of men.

Discussion

As mentioned in the introduction considerable uncertainty prevails as to the mechanism of the disulfiram-ethanol reaction. Several investigators have provided evidence against the assumption of an increased acetaldehyde concentration in the blood as the central factor involved in the reaction (RAIT 1955 & 1956 PERMAN 1962 CASIER & POLET 1958 STRØMME 1964). From his experiments with rabbits pretreated with disulfiram PERMAN (1962) concluded that the ethanol induced fall in blood pressure must be due to some

Table 6

Determination of total kininogen kininogen fractions and rate of release f kinin in plasma from untreated males and from males treated with disulfiram.

Kinin parameter preparations from 3 males treated with disulfiram were prepared and tested separately (B), while the corresponding control preparations were made from the pooled plasma from 4 healthy untreated males (A).
Kinin released per ml plasma was calculated as μg bradykinin (I, II, III). For further details see text.

	Kinin system parameter		A	B
I	Total kininogen		5.0	5.0
				5.0
				5.0
	Mean			5.0
II	The kininogen fraction activated by plasma kallikrein		1.8	2.3
				1.7
				1.7
	Mean			1.9
III	The kininogen fraction activated by padutin (hog pancreas kallikrein)		4.0	3.5
				4 6
				4.6
	Mean			4.2
IV	Time in minutes corresponding to 66 % kinin release caused by padutin	0.35 units per ml plasma	19	21
				20
				26
		Mean		22
		0.70 units per ml plasma	21	18
				21
				26
		Mean		22

unknown blood-borne substance acting on the peripheral vessels. The plasma kinins which are readily released from the plasma kininogens by plasma kallikrein have properties which make them hypothetical candidates for the blood pressure effect. It seemed of interest to examine the possible effect of ethanol and of disulfiram on the factors involved in the plasma kinin system. Acetone is known to activate plasma kallikrein (KRAUT *et al* 1933 and several others) and a similar effect might be obtainable with ethanol. r r

too, might interfere with some factors in the plasma kinin system, for example by inhibiting the enzymatic destruction of kinin. DYRUD *et al* (1965) observed in *in vitro* experiments that disulfiram inhibited human erythrocyte kininase, but not human plasma kininase. ELDJARN (1950) and STRØMME (1964) on the other hand, showed that disulfiram in the organism is readily reduced to diethyldithiocarbamate, and ELDJARN suggested that diethyldithiocarbamate and not disulfiram was the active substance. The results in the present paper clearly show that diethyldithiocarbamate is a potent inhibitor of plasma kininase. The experiments also gave evidence of a kinin-releasing effect of ethanol, although the effect of ethanol alone was only significant in very high concentrations. The results of the experiments with ellagic acid (which is known to activate plasma kallikrein) *and* ethanol showed that ethanol in small concentrations potentiated the kinin-releasing effect of ellagic acid. This might indicate that ethanol, at any rate, has some activating effect on the plasma kinin system. It seems possible that ethanol under normal conditions is not able to increase the plasma kinin level to an extent which will cause a hypotension comparable to that of the human disulfiram-ethanol reaction. The disulfiram treatment, however might interfere with one or more of the numerous factors involved in the plasma kinin system, making the kinin releasing effect of ethanol significant. It should be mentioned (unpublished experiments) that the hypotensive effect of bradykinin in rats was specifically potentiated by diethyldithiocarbamate.

The comparative determinations of different factors of the kinin system in the plasma from males treated with disulfiram and from untreated males, however did not provide any evidence of differences between the two groups. Since the *in vitro* experiments showed that diethyldithiocarbamate is a relatively potent inhibitor of human plasma kininase, and as disulfiram is readily reduced to diethyldithiocarbamate in the organism (ELDJARN 1950, STRØMME 1964) it was somewhat surprising to find that the kininase preparations from the disulfiram treated males and from the untreated males had the same activity. The possibility that the technique used for the plasma kininase determinations may mask some possible differences should be further examined.

REFERENCES

Asmussen, E., J. Hald & V. Larsen: The pharmacological action of acetaldehyde on the human organism. *Acta pharmacol. et to icol.* 1948, 4 311–320.

Briseid, K., F. C. Arntzen & O. K. Dyrud. Studies on the release of kinin by acetone in human plasma. *Acta pharmacol. et to icol.* 1968a, 26 402–412.

Briseid, K., O. K. Dyrud & F. C. Arntzen: Determination of prekallikrein in human plasma. *Acta pharmacol. et to icol.* 1968b 26, 395–401.

Briseid, K., O K. Dyrud & S. F Rinvik. *In vitro* estimation of the rate of release of kinin in human plasma. *Acta pharmacol. et toxicol.* 1967 25, 201–213

Casier H. & H. Polet: Influence du disulfiram (antabus) sur le métabolisme de l alcool éthylique marqué chez la souris. *Arch. int Pharmacodyn.* 1958, 113, 439–496.

Dyrud, O. K., & F Rinvik & K. Briseid Jensen. Effects of inhibitors on the *in vitro* inactivation of bradykinin by various kininases. *Acta pharmacol. et toxicol* 1965, 23, 235– 49

Eldjarn, L. The metabolism of tetraethyl thiuramdisulphide (antabus, aversan) in man investigated by means of radioactive sulphur *Scand. J clin. Lab. I nvest* 1950, 2, 202–208.

Graham, W D. *In vitro* inhibition of liver aldehyde dehydrogenase by tetraethylthiuramdisulphide. *J Pharm. Pharmacol.* 1951, 3, 160–168.

Hald, J & E. Jacobsen. The formation of acetaldehyde in the organism after ingestion of antabuse (tetraethylthiuramdisulphide) and alcohol. *Acta pharmacol. et toxicol* 1948, 4 305–310

Hald, J E. Jacobsen & V Larsen. The sensitizing effect of tetraethylthiuramdisulphide (antabuse) to ethylalcohol. *Acta pharmacol. et toxicol.* 1948, 4 285–296.

Hald, J E. Jacobsen & V Larsen. The rate of acetaldehyde metabolism in isolated livers and hind limb of rabbits treated with antabuse (tetraethylthiuramdisulphide) *Acta pharmacol. et toxicol.* 1949 5 298–308.

Kjeldgaard, N. O. Inhibition of aldehyde oxidase from liver by tetraethylthiuramdisulphide (antabuse) *Acta pharmacol. et toxicol.* 1949 5 397–403.

Kraut, H., E. K. F ey & E. Werle. Über den Nachweis und das Vorkommen des Kallikreins im Blut. VIII Mitteilung über Kallikrein. *Hoppe Seyler s Z f physiol Chem.* 1933, 222, 73–99

Larsen, V The effect on experimental animals of antabuse (tetraethylthiuramdisulphide) in combination with alcohol. *Acta pharmacol. et toxicol* 1948, 4 321–332.

Perman, E. S. Studies on the antabuse-alcohol reaction in rabbits. *Acta physiol. scand.* 1962, 55, suppl. 190.

Raby K. *Kliniske og experimentelle undersögelser ove antabus-alkohol reaktionen.* Thesis. Copenhagen 1955

Raby K. The antabus-alcohol reaction. Summary of clinical and experimental investigations. *Dan. med Bull.* 1956, 3, 168–171.

Rinvik, S. F O K. Dyrud & K. Briseid: Determination of plasma kininogen, plasma kininase and erythrocyte kininase. *Acta pharmacol. et toxicol* 1966, 24 169–178.

Strömme, H., *Studies on metabolism and biochemical effects of tetraethylthiuramdisulphide (disulfiram) and diethyldithiocarbamate* 1964, Universitetsforlaget, Oslo, Norway

from 52 to 54 % (4 determinations). For recovery of NA and DA see PERSSON & WALDECK (1968). No corrections for recovery were made.

L-DOPA 2, 5 6-^{3}H with the specific activity 10–30 ci/mmole, in an aqueous solution containing 2 % ethanol, was obtained from The Radiochemical Centre, Amersham, England. L-DOPA labelled with tritium at a high specific activity is not very stable. The material we used was regularly checked by radiopaper chromatography isopropanol/2 N-HCl, 65/35 v/v 18 hrs ascending. From time to time various amounts of a radioactive impurity appeared. A typical scan is shown in fig. 1. The concentration of the impurity increased with the period of storage (+ 4°). The rate of decomposition by self-radiolysis can be reduced by storing the solution at − 196 (EVANS 1966). The impurity found here was not clearly revealed by the following chromatographic systems: a) n-butanol/acetic acid/water (12/3/5 v/v), b) n-butanol saturated with 2 N hydrochloric acid (equal volumes) and c) isopropanol/ethanol/N hydrochloric acid (75/75/50) all ascending[1]. This situation calls for caution and may indicate that the impurity is structurally related to DOPA, presumably a hydroxylation product e. g. 6-hydroxy-DOPA or dihydroxyphenylserine.

Specificity

Six rats, divided into two groups, received 5 µg/kg ^{3}H-DOPA intravenously. One group was sacrificed at 30 min. and the other 16 hrs later. These extraction and separations on alumina and ion-exchange columns were performed as described above. The eluates containing DOPA and MTyr respectively were evaporated at 25 to a small volume under reduced pressure and then applied to paper and chromatographed in isopropanol/2 N hydrochloric acid, 65/35 v/v. After spraying with diazotated p-nitroaniline, the papers were cut into 1 cm transverse strips which were soaked in scintillation solution. The radioactivity of each strip was determined. The result is shown in fig. 2. In all cases the radioactivity followed the respective carrier spot. Sixteen hours after the injection, however ^{3}H DOPA had reached too low levels for identification.



Fig. 1. Radiochemical impurity of L-DOPA-2, 5 6-^{3}H. An aliquot of ^{3}H-DOPA was mixed with carrier DOPA and 2 N-HCl and applied on paper. The paper was developed in isopropanol/2 N-HCl, 65/35 v/v 18 hrs ascending. After staining with diazotised p-nitroaniline the paper was scanned for radioactivity. The figure shows the recording, the cross hatched area indicating the position of the carrier spot.

1) Until now the manufacturers in their analysis certificate have referred to these three systems only.

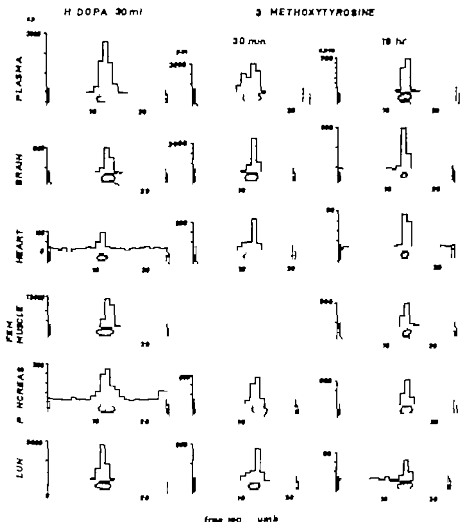


Fig. 2. Identification of ³H-DOPA and ³H-methoxytyrosine in different tissues after the intravenous administration of ³H-DOPA. Rats were given 5 μg/kg ³H-DOPA intravenously and were killed 30 min. or 16 hrs thereafter. Tissue extraction and ion-exchange chromatography was performed as described in the text. The eluates were reduced to small volume and applied on paper which was then developed in isopropanol/2 N-HCl, 65/35 v/v 18 hrs ascending. After staining, the paper was cut into 1 cm transversal strips, each strip being counted in liquid scintillation counter. Each sample derives from the pooled tissues of 3 animals. The shaded areas show the positions of respective carrier spots.

Results

The concentrations of ³H DOPA, ³H-DA and ³H NA in blood plasma and various tissues 15 min., 1 and 2 hrs after the intravenous admin

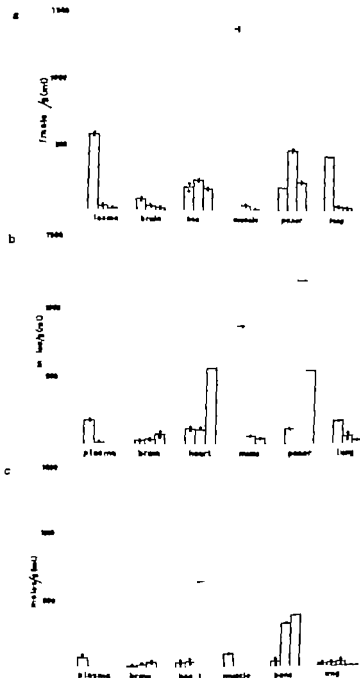


Fig. 3. Levels of ^{3}H-DOPA, ^{3}H-dopamine and ^{3}H-noradrenaline in different tissues after the intravenous administration of ^{3}H DOPA. Rats were given 5 µg/kg ^{3}H DOPA intravenously. The animals were killed a: 15 min., b: 1 hr and c: 2 hrs thereafter. The tissues were taken separately for analysis of the labelled compounds. Each dot represents the pooled samples of 2 rats. The three bars of each block represent from the left ^{3}H-DOPA, ^{3}H-dopamine and ^{3}H-noradrenaline respectively.

of 5 µg/kg ^{3}H DOPA are shown in fig. 3 In a pilot study we found about 25,000 fmoles/ml[1] plasma of ^{3}H DOPA 15 sec. after the injection. Fifteen minutes later the plasma concentration had decreased to about 1/50th of this value. At this time interval the ^{3}H DOPA concentration in the femoral muscle was about twice that in the plasma and in the lung it was of the same order of magnitude as in the plasma. The brain, heart and pancreas had a lower ^{3}H DOPA concentration, 1/7th, 1/7th and 1/3rd of the plasma concentration respectively 15 min. after the injection. During the next two hours ^{3}H DOPA appeared to decrease almost uniformly in the different tissues, the decrease in the plasma and femoral muscle being possibly some what faster

The accumulation of ^{3}H-NA and ^{3}H DA was relatively low in brain, femoral muscle and in the lung. In the heart and pancreas it was about ten times higher Fifteen minutes after the injection of ^{3}H DOPA there were about equal amounts of ^{3}H-NA and ^{3}H DA in the heart. During the next 2 hrs the ^{3}H-NA in this organ increased whereas ^{3}H DA decreased. In the pancreas, ^{3}H-DA reached a very high concentration (about 1,200 fmoles/g) one hour after ^{3}H-DOPA.

Attempts were also made to estimate the half life of ^{3}H DOPA, ^{3}H DA and ^{3}H-NA from determinations at 2, 4 8 and 16 (in some experiments 48) hrs respectively after the administration of the precursor (tables 1 and 2) As in the condition found during the first two hours, ^{3}H DOPA appeared to disappear more rapidly from the plasma than from the brain and heart. In the brain, the half lives of ^{3}H DOPA and ^{3}H NA were about equal, and for ^{3}H DA somewhat less. ^{3}H-DA in the femoral muscle and lung and ^{3}H-NA in the pancreas disappeared with a half life of around twenty hours. For the remainder of the material half-lives could not be demonstrated. However ^{3}H-NA in the heart remained at a high level throughout the experiment. Retention of larger amounts of ^{3}H DOPA could not be observed in any of the tissues examined.

During the first hour after the administration of ^{3}H DOPA the metabolite ^{3}H-MTyr increased in all the tissues and in the plasma. After 1 hr it was in general 2–6 times higher than the corresponding ^{3}H DOPA levels (table 3). In the pancreas it was about 20 times higher This tissue also accumulated 5–10 times more ^{3}H MTyr than did any of the other tissues or the plasma. The elimination of this metabolite appeared to occur very slowly From 8 to 48 hrs after the injection of ^{3}H-DOPA there were still large amounts of ^{3}H MTyr as compared with the content of ^{3}H-catechols. One exception is the heart which contained more ^{3}H NA.

---

[1] fmole = femtomole = 10^{-15} moles.

Table 1

Disappearance of ^{3}H DOPA, ^{3}H-dopamine and ^{3}H-noradrenaline in different tissues after the intravenous administration of ^{3}H-DOPA. Rats were given 5 µg/kg ^{3}H DOPA intravenously The animals were killed at various time intervals thereafter and the levels of ^{3}H DOPA, ^{3}H-dopamine (^{3}H DA), and ^{3}H-noradrenaline (^{3}H-NA) in the plasma and in the different tissues were determined. The values, derived from the pooled samples of 2 animals, are in fmoles/g and ml respectively

	Time interval hrs															
	2			4			8			16			48	
	^{3}H-DOPA	^{3}H-DA	^{3}H-NA	^{3}H-DOPA	^{3}H-DA	^{3}H-NA	^{3}H-DOPA	^{3}H-DA	^{3}H-NA	^{3}H-DOPA	^{3}H-DA	^{3}H-NA	^{3}H-DA	^{3}H-NA
Plasma	36	4	2	22	4	5	16	3	4	–	2	3		
	81	4	2	38	5	6					6	14		
	97	10	9	47	6	6								
Brain	9	13	30	4	7	18	6	5	14	2	3	5		
	17	21	31	4	8	19		5	19		1	9		
	17	22	61	8	9	31								
Heart	76	32	437	18	12	350	8	12	689	5	8	562		
	40	37	754	23	17	351	20	17	756		18	927		
	51	66	767	28	18	765								
Femoral muscle	104	12	12	22	8	23	8	10	37	2	6	21	2	5
		9	20	34	12	15					11	26		
Pancreas	24	328	262	21	10	268	20	12	365	1	10	168	2	47
	59		525	24	25	273					37	205		
Lung	27	16	11	21	14	29	12	13	19	1	11	13	4	4
	11	27	48	11	18	34					21	36		

Table 2

Half lives of ³H-DOPA, ³H-dopamine and ³H-noradrenaline in different tissues from 2 hrs after the intravenous administration of ³H-DOPA. The values are calculated from the data in table 1. The half lives in hours and the P value for the linear correlation of the logarithm of the concentration to the time are shown. N S. = not significant ($P > 0.05$).

	³H-DOPA	³H-dopamine	³H-noradrenaline
Plasma	2.9 $P < 0.05$	9.5 N S.	− 17.8 N S.
Brain	5.5 $P < 0.01$	4.4 $P < 0.001$	5.9 $P < 0.001$
Heart	4.8 $P < 0.001$	9.6 $P = 0.05$	− 34 N S.
Femoral muscle ..	2.7 N S.	20 $P < 0.001$	− 10.8 N S.
Pancreas	2.8 N S.	8.4 N S.	16 $P < 0.001$
Lung ...	3.5 N. S.	21 $P < 0.01$	17.3 N S.

Table 3

Levels of ³H methoxytyrosine in different tissues after the intravenous injection of ³H-DOPA. Rats were given 5 µg/kg ³H-DOPA intravenously. The animals were killed at various time intervals thereafter and ³H-methoxytyrosine in the plasma and the different tissues was determined. The values, derived from the pooled samples of 2 animals, are in fmoles/g and ml respectively

	Time interval hrs						
	¼	1	2	4	8	16	48
Plasma	310	397	277	128	−	117	26
	408	449	638	154		−	−
Brain ..	45	96	141	69	182	73	22
	72	193	224	70	−	−	−
Heart ..	51	387	129	86	214	22	28
	116	449	458	129		−	−
Femoral muscl ...	150	416	152	61	381	35	25
	153	690	289	118			−
Pancreas	428	2297	2520	223	1540	628	133
	1263	2521	−	694	−	−	−
Lung	214	185	107	39	289	129	22
	217	521	147	88	−	−	−

REFERENCES

Alm, P B. Ehinger & B. Falck: Histochemical studies on the metabolism of L-DOPA and some related substances in the exocrine pancreas. *Acta physiol. scand.* 1969 76 106–120.

Anton, A. H. & D. F Sayre: The distribution of dopamine and dopa in various animals and a method for their determination in diverse biological material. *J Pharm. Exp. Ther* 1964 145 326–336.

Bartholini, G & A. Pletscher: Cerebral accumulation and metabolism of ^{14}C-DOPA after selective inhibition of peripheral decarboxylase. *J Pharm Exp. Ther* 1968, 161 14–20.

Bertler A., A. Carlsson & E. Rosengren. A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. *Acta physiol. scand.* 1958, 44 273–29?

Bertler A., B Falck, Ch. Owman & E. Rosengren: The localization of monoaminergic blood-brain barrier mechanisms. *Pharm. Rev* 1966, 18, 369–385.

Burack, W R. & P R. Draskóczy: The turnover of endogenously labelled catecholamine in several regions of the sympathetic nervous system. *J Pharm. Exp. Ther* 1964 144 66–75

Carlsson, A. & B. Waldeck: On the role of the liver catechol-O-methyl transferase in the *metabolism of circulating catecholamines. Acta pharmacol. et* toxicol. 1963, 20 47–55

Constantinidis, J J C. de la Torre, R. Tissot & F Geissbuhler: La barrière capillaire pour la dopa dans le cerveau et les différents organes. *Psychopharmacologia* (Berlin) 1969 15, 75–87

Evans, E. A. Control of self-irradiation decomposition of tritium-labelled compounds at high specific activity *Nature* 1966, 209 169–171.

Ferrini, R. & Glässer A.. *In vitro* decarboxylation of new phenylalanine derivatives. *Biochem Pharmacol.* 1964 13, 798–800

Hagemann, E. & G Schmidt: *Ratte und Maus Versuchstiere in der Forschung.* Walter de Gruyter & Co., Berlin 1960, p. 124

Persson, T Central and peripheral catecholamine turnover studied by means of ^{3}H-DOPA and ^{3}H-tyrosine. *Acta pharmacol. et toxicol.* 1969 27 397–409

Persson, T & B Waldeck: The use of ^{3}H DOPA for studying cerebral catecholamine metabolism. *Acta pharmacol. et toxicol* 1968, 26, 363–372.

Persson, T & B. Waldeck. Some problems encountered in attempting to estimate the catecholamine turnover using labelled tyrosine. *J Pharm. Pharmacol.* 1970, 22, 473–478.

von Studnitz, W Metodische und klinische Untersuchungen über die Ausscheidung der 3-methoxy-4-hydroxy-Mandelsäure im Urin. *Scand. J Clin. Lab. Invest.* 1960, 12, Suppl. 48

Symchowicz, S., C. Korduba, J Veals & I L. A. Tabachnick: Norepinephrine biosynthesis in the rat pancreas. *Biochem. pharmacol.* 1968, 17 2313–2317

Tissot, R., G Bartholini & A. Pletscher: Drug induced changes of extracerebral dopa *metabolism in man. Arch Neurol.* 1969 20 187 190.

Udenfriend, S. & P Zaltzman-Nirenberg: Norepinephrine and 3,4-dihydroxyphenylethylamine turnover in guinea pig brain *in vivo. Science* 1963, 142, 394–396.

Waldeck, B.. Preparation of aqueous samples for liquid scintillation counting with the aid of freeze-drying. *Acta physiol. scand.* 1968, 73, 9–10A.

Vendsalu, A.. Studies on adrenaline and noradrenaline in human plasma. *Acta physiol. scand.* 1960, 49 suppl. 173

Acta pharmacol. et toxicol 1970 28, 477–483

From the Department of Pharmacology and Toxicology School of Veterinary Medicine, Free University Berlin, Germany

Time Course of the Demethylation of Trimethadione

By

H.-H. Frey and R. Schulz

(Received May 13 1970)

Abstract. A gas chromatographic method for the determination of trimethadione in the serum is described. By this method, the time course of the demethylation of trimethadione to dimethadione, the latter being determined by UV-spectrophotometry was followed in mice and dogs. For the demethylation of trimethadione, half-life of 45 min. in mice and of 8 hours in dogs was found. The dimethadione formed is excreted extremely slowly half-lives were about 30 hours in mice and 50–80 hours in dogs. During chronic treatment dimethadione accumulates: with daily doses of 22–23 mg/kg trimethadione dimethadione concentrations of 175 to 225 µg/ml, while with doses of 64 mg/kg concentrations of about 400 µg/ml were reached in dogs. Under these conditions, unmetabolized trimethadione does not contribute more than 15 per cent maximally to the total anticonvulsant effect of treatment, and the determination of the serum concentrations of dimethadione is therefore considered sufficient for control of therapy

Key words. Trimethadione – drug metabolism anticonvulsant effect.

In 1952, 1953 and 1954 Butler and his group showed that trimethadione (3,5,5-trimethyloxazolidine 2,4-dione) is almost quantitatively demethylated to 5,5-dimethyloxazolidine-2,4-dione (dimethadione), and suggested that the latter might play a considerable role in the anticonvulsant effect of trimethadione. An anticonvulsant effect of the metabolite has been shown both in animals (Taylor *et al.* 1956) and in man (Chamberlin *et al.* 1965)

Two questions remained to be answered 1 whether unmetabolized trimethadione had an anticonvulsant effect of its own and how strong this was in relation to that of the metabolite, and 2. was it not possible to determine the role both compounds played in the clinical effect, since no sensitive method for the determination of trimethadione was available. With regard to the first of these questions, one of us (Frey 1969) has recently shown by indirect approach, that on a molar base trimethadione has 1.25

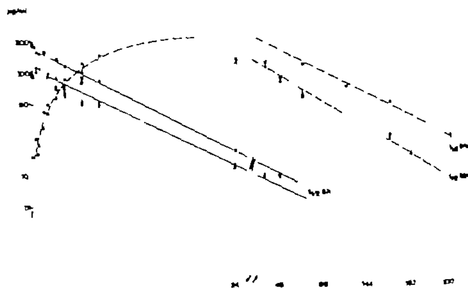


Fig. 2. Semi-logarithmic plot of the serum concentrations of trimethadione (closed symbols) and dimethadione (open symbols) in 3 dogs after intravenous injection of 100 mg/kg trimethadione. Circles, triangles and squares are used for the respective dogs.

viously higher than that for the metabolite, which, in agreement with former experiments of BUTLER (1953) and WADDELL & BUTLER (1959) was found in one dog to be 40 per cent of the body weight. The higher V_d of trimethadione is considered to be an expression of a higher lipophility particularly since the highest values were found in two dogs who were moderately to extremely obese.

Within 4–6 hours after the injection dimethadione concentrations began to exceed those of trimethadione, the maximum concentrations of the metabolite being reached after 24 to 32 hours. The fall in the concentration of the dimethadione occurred with a half life of 58 hours in two dogs and of 66 hours in the third dog.

The finding that serum concentrations after intravenous injection of trimethadione remained constant for the first 60–90 min. deserves some comment. A possible explanation may lie in the processes of distribution during this period, but it is difficult to see how for example, the body fat would be able to trap greater amounts of the injected drug within this time period when the relatively bad blood supply of this tissue is considered. Another possibility would be that the high concentration of trimethadione immediately after the intravenous injection exerted an inhibitory effect on the demethylating enzyme in the liver. This view finds some support in the fact that calculation of the serum concentration of dimethadione in mice with the usual pharmacokinetic equations (DOST 1968), using the k_e value from the exponential decline

of trimethadione and correcting for the different volumes of distribution gave higher values than those found experimentally in the first hour after injection. The possibility of a biphasic effect of trimethadione on drug metabolism is mentioned by CONNEY (1967)

After *oral administration* to dogs in single doses of 90–110 mg/kg, the concentration of trimethadione in the serum rose rapidly the maximal concentrations being reached after one hour in two dogs and after 3 hours in the third dog (fig. 3) The trimethadione levels then fell and were already exceeded by those of dimethadione between 4 and 8 hours.

Our dog experiments with *chronic oral treatment* were of special interest in the question of the role played by the drug and the metabolite in the clinical anticonvulsant effect during continued treatment. Since trimethadione is demethylated to dimethadione relatively rapidly and the latter is not further metabolized and excreted so slowly that it accumulates to a considerable extent, it was expected that under steady state conditions high levels of dimethadione and comparatively low levels of trimethadione would be present in the organism. This was borne out by our dog experiments in which, dependent on the dose, steady state concentrations of between 175 and 400 µg/ml dimethadione were reached at the end of the first week of treatment (fig. 4). The trimethadione concentrations, on the other hand, remained practically constant throughout the whole duration of the experiment: they usually amounted to 5–10 µg/ml in the morning, i. e., before the daily dose was given, and rose dose-dependently to maximal values of 30–50 µg/ml after two to four hours. The steady state level reached with dimethadione was in good



Fig. 3. Time course of serum concentrations of trimethadione (closed symbol) dimethadione (open symbols) in 3 dogs after oral administration of about trimethadione. Circles, triangles and squares are used for the respect.

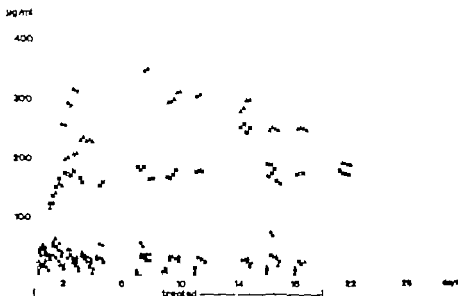


Fig. 4 Serum concentrations of trimethadione (closed symbols) and dimethadione (open symbols) in 3 dogs treated with daily doses of trimethadione during three weeks. The daily dose was 22–23 mg/kg in the dogs symbolized by triangles and squares, and 64 mg/kg in the dog symbolized by circles. For further explanation see text.

agreement with the values predicted from application of standard pharmacokinetic equations (GOLDSTEIN *et al.* 1968) using a V_d of 40 per cent of body weight, the k_e values being determined after cessation of treatment and the dimethadione equivalent of the daily dose of trimethadione. After the end of treatment, dimethadione concentrations *fell with half-lives of 48, 72 and 77* hours respectively in the three dogs.

Using the potency ratio between trimethadione and dimethadione which had been calculated to 1.25 on a molar and 1 13 on a weight basis in favour of trimethadione (FREY 1969) we were able to calculate the percentage both ω contribute to the total clinical effect. Beginning from the third day of treatment, the contribution of trimethadione to the "total anticonvulsant potency of the medication averaged only 2–5 per cent in the morning and 12–15 per cent at the time of maximal serum concentrations (2 and 4 hours after the daily dose had been given) This relation remained remarkably constant for the whole duration of the experiment.

Thus, dimethadione must be considered to be mainly responsible *for the* clinical anticonvulsant effect. This conclusion confirms the early *suggestion* of BUTLER *et al.* (1952, 1953 and 1954) and it also gives an explanation *for* the clinical observation that the control of epileptic seizures is first complete after two to four days of treatment (GOODMAN *et al* 1946) i e this time is

necessary for dimethadione to accumulate in sufficient concentrations in the body The effective level of dimethadione in man is between 500 and 1000 µg/ml according to the results of CHAMBERLIN *et al* (1965) and JENSEN (1962). For the clinical control of medication, a control of dimethadione serum concentrations, which is easily achieved by UV-spectrophotometry should be adequate.

Acknowledgements

The experiments were performed with the skilful technical assistance of Mr. Eberhard Manz and Miss Heide Wiemann

REFERENCES

Butler T C., D. Mahaffee & C. Mahaffee: Metabolic demethylation of 3,5,5-trimethyl-2,4-oxazolidinedione (trimethadione, tridione). *Proc. Soc exp Biol.* (N Y.) 1952, 81, 450–452.

Butler T C.: Quantitative studies of the demethylation of trimethadione (tridione). *J Pharmacol. exp Ther* 1953, 108, 11–17

Butler T C. & W J Waddell. The role of the liver in the demethylation of N-methyl derivatives of hydantoin and 2,4-oxazolidinedione. *J Pharmacol. exp. Ther* 1954, 110, 241–243

Chamberlin, H. R., W J Waddell & T C. Butler A study of the product of demethylation of trimethadione in the control of petit mal epilepsy *Neurology* (Minneap.) *1965* 15 449–454.

Conney A. H.. Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev* 1967 19 317–366.

Dost, F H.. *Grundlagen der Pharmakokinetik*. 2. Aufl., Georg Thieme-Verlag, Stuttgart 1968, S. 36–47

Frey H-H.. Determination of the anticonvulsant potency of unmetabolized trimethadione. *Acta pharmacol. et toxicol.* 1969 27 295–300

Goldstein, A., L. Aronow & S. M. Kalman. *Principles of drug action. The basis of pharmacology* Hoeber New York City N. Y 1968, p 292–309

Goodman, L. S., J E. P Toman & E. A. Swinyard. The anticonvulsant properties of tridione; laboratory and clinical investigations. *Amer J Med.* 1946, 1, 213–228.

Jensen, B. N.. *Trimethadione in serum of patients with petit mal epilepsy Dan. med. Bull.* 1962, 9 74–79

Taylor J D I D Davis & R. K. Richards: Duration of anticonvulsant action of trimethadione and some demethylated oxazolidinediones against pentylenetetrazole in mice. *Fed. Proc.* 1956, 15, 491.

Waddell W J & T C. Butler: Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxazolidinedione *(DMO). Application to skeletal muscle of the* dog *J clin. Invest* 1959 38, 720–729

Acta pharmacol et toxicol 1970 28, 484–492.

From the Section of Toxicology
Department of Pharmacology (Professor Jens Schou, M. D.),
University of Copenhagen, Denmark

The Urinary Excretion of Enibomalum (NFN) in Man after Administration of Anaesthetic Doses

By

A. Ravn-Jonsen

(Received May 15, 1970)

Abstract: Anaesthetic doses of enibomal (500–1000 mg intravenously) were administered to eight patients and the urine subjected to thin-layer chromatography. Four metabolites were detected, but no enibomal. 5-acetonyl-5-isopropylbarbituric acid (VIII) was demonstrable for up to 6 days, and another metabolite, 1 methyl-5-(2'-oxo-3'-hydroxypropyl)-5-isopropylbarbituric acid (VI), was almost equally long lasting. 1-methyl 5-acetonyl-5-isopropylbarbituric acid (II) and ibomal (NFN) (VII) were present more sporadically and only during the first 2–3 days. VIII was identified by means of IR-spectrophotometry. The identity of VI was confirmed on three TLC-systems by comparison with synthetic VI. The alteration in the UV-spectrum after exposure to ammonia was similarly used for identification. The identity of II was determined by TLC and UV spectrophotometry while VII was determined exclusively by its location on the TLC-system. Quantitative analysis of the urine from four patients, showed VI and VIII to be the main excretion products (6–9 per cent and 2–7 per cent of the dose given). II constituted no more than 0.3–0.9 per cent, while VII occurred in such a small amount that quantitative determination was impossible. The investigation showed that enibomal is deposited in the organism, but not whether its short-term action is due to deposition or to rapid metabolism.

Key-words: Enibomal – barbiturates – metabolism – 1-methyl-5-(2'-oxo-3'-hydroxypropyl)-5-isopropylbarbituric acid.

There are two possible mechanisms for the metabolism of N-substituted barbituric acid compounds *in vivo*. One is oxidation of one of the substituents at the carbon atom in position 5 in the barbituric acid ring, and the other is de-alkylation at the nitrogen atom. Which of these two possibilities predominates depends on the substituents at the carbon atom in position 5. 1-methyl 5-β-bromallyl 5-isopropylbarbituric acid (enibomal *NFN*) may also be subject to hydrolytic liberation of the halogen atom by the double bond with production of a ketone (Halberkann & Reiche 1927). This barbituric

acid compound can thus theoretically follow the metabolic pathways shown in fig. 1.

FREY (1959), on paper-chromatographic analysis of the renal clearance products from dogs, found that demethylation and hydrolysis occur. FREY (1959) also showed that the excretion products are detectable for several days after administration, even though enibomal is a barbituric acid compound with a short term action.

To study whether the conversion proceeds along the same metabolic pathways in man as in dogs, we followed the excretion for up to 10 days after intravenous injection of anaesthetic doses. The investigation was restricted exclusively to the compounds I, II, III, V, VI, VII, VIII, and XI (fig. 1). To get an impression of the influence of the detected compounds on the elimination of enibomal, we also subjected the metabolites found to quantitative estimation.

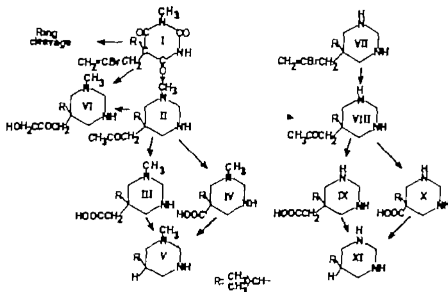


Fig. 1. The theoretical metabolic pathways of enibomal.
I Enibomal = 1-methyl-5-β-bromoallyl-5-isopropylbarbituric acid
II 1-Methyl-5-acetonyl-5-isopropylbarbituric acid
III 1-Methyl-5-carboxymethylene-5-isopropylbarbituric acid
IV 1-Methyl-5-carboxy-5-isopropylbarbituric acid
V 1-Methyl-5-isopropylbarbituric acid
VI 1-Methyl-5-(2'-oxo-3'-hydroxypropyl)-5-isopropylbarbituric acid
VII Ibomal = 5-β-bromoallyl-5-isopropylbarbituric acid
VIII 5-Acetonyl-5-isopropylbarbituric acid
IX 5-Carboxymethylene-5-isopropylbarbituric acid
X 5-Carboxy-5-isopropylbarbituric acid
XI 5-Isopropylbarbituric acid

Material and Methods

The patients, all operated on for a slipped disc, were anaesthetized by intravenous injection of 500–1000 mg narcodorm® (enibomal sodium NFN), followed by gaseous anaesthetics (e. g. dinitrogenum oxidum and halothanum NFN). Urine collected during the period from the close of the operation up til 7 o clock on the next morning, was designated as the 1st 24-hour urine sample. Thereafter the collection of the 24-hour urine sample was concluded every morning at 7 o'clock. The patients were given no barbituric acids before or after the operation, and other drugs that might interfere with the analytical method were avoided.

At least one 24-hour urine sample was collected before the start of the experiment to serve as a blank. All the samples were stored at − 20 until the commencement of the analysis.

Synthesis of reference samples.

Preparation of the compounds II, III and V has been described previously (RAVN-JONSEN & HEDIN 1968).

VIII 5-acetonyl-5-isopropylbarbituric acid: 3.4 g isopropylbarbituric acid is dissolved in an equivalent quantity of 30 % sodium hydroxide solution, and 3 g bromacetone is added. The mixture is left for 24 hours at room temperature under continuous stirring. The precipitate is filtered off, and purified by dissolving in ether and extracting with buffer pH 10.0. The aqueous phase is made acid and extracted with ether. After evaporation of the ether the precipitate is washed twice with ether. The yield is about 10 % of almost colourless crystals with a melting point of 260–1 (Found after drying at 50°/2 mm C: 52.81 % H. 6.32 % N 12.20 % calculated, C: 53.09 % H. 6.24 % N 12.39 %)

VI 1-methyl-5-(2'-oxo-3'hydroxy-propyl)-5-isopropylbarbituric acid. 6.2 g enibomal (0.02 mol) is dissolved in an equivalent quantity of 1 N sodium hydroxide. To this is added 1.05 g potassium permanganate (0.007 mol) dissolved in 80 g water. The addition proceeds slowly and under continuous stirring. After the addition has been concluded the precipitate is filtered off, and the excess of permanganate in the filtrate is removed with sulphite. The aqueous phase is made acid and extracted with a large volume of ether. The ether phase is dried and evaporated to a suitable volume. It is then extracted, first with ¼ volume of phosphate buffer pH 7.5 and next, with ¼ volume of phosphate buffer pH 10.0. The latter buffer is made acid and extracted with a large volume of ether. After evaporation of the ether, the residue is recrystallised in cyclohexane. The yield is about 10 % of colourless crystals with a melting point of 141–3. The compound displays, within the UV-range, the absorption curves characteristic of N-substituted barbituric acids (STUCKEY 1941). The infrared spectrum shows two strong bands at 3500 cm^{-1} and 1050 cm^{-1} indicating a primary alcohol group. (Found after drying at 50 /2 mm: C. 51.52 % H. 6.37 % N 10.83 % calculated, C: 51.54 % H 6.30 % N 10.93 %)

Analytical methods

A. *Analysis for free I II VI VII and VIII*

100 ml of 24-hour urine specimens are extracted three times with 3–4 volumes of ether in a separating funnel after the addition of 8 N hydrochloric acid to a pH of about 2. The combined and dried ether phases are evaporated to 40 ml and purified by extraction with 10.00 ml of phosphate buffer pH 7.5. Extraction with 10.00 ml of 0.1 M phosphate buffer pH 12.0 (phase A) then follows.

Phase A is re-extracted with ether (three times 30 ml) at pH 2, and the combined ether phases are evaporated to dryness. One tenth of the residue is drawn for qualitative

analysis and subjected to chromatography in solvent A (table 1). After development the chromatogram is studied in UV-light of a wavelength 256 nm, both before and after exposure to vaporised ammonia. Regions that absorb after exposure to ammonia are marked. The chromatogram is then sprayed with a mercuric sulphate-diphenylcarbazone reagent (SMITH 1960).

For quantitative analysis the residue from evaporation is divided into two aliquots (1/10 and 9/10). They are both chromatographed in solvent A. Both aliquots are analysed in UV-light, *but only the smaller one is sprayed. Dependent on the number of* mercury-positive spots, the larger aliquot is divided into zones, which are eluted with methanol. The methanol is removed by evaporation. The residue is dissolved in 12 ml of ether and extracted, first with 3.00 ml of phosphate buffer pH 10.0 (phase B) and then with 3.00 ml of phosphate buffer pH 12.0 (phase C). Phase B is used for quantitative spectrophotometric determinations of *II, VI* and *VIII* and phase *C* of *I* and *VII*. The spectrum is recorded for the whole range of 200–350 nm in order to study the purity of the solutions.

B. *Analysis for free and glycuronic-acid-conjugated II III V VI VIII and XI*

50–100 ml of 24-hour urine specimen is treated with 10,000 units of glycuronidase (Sigma®) at pH 5 for 3 hours at 37° The urine is then extracted as described above. The ether phase is extracted, first with 10.00 ml of 0.1 M phosphate buffer of pH 5.5 (phase D), then with 10.00 ml of buffer of pH 7.5 (phase E), and finally with 10 00 ml of buffer of pH 10.0 (phase F).

III and XI Phase D is extracted three times with 3–4 volumes of ether at pH 1 3. The combined extracts are evaporated, and three aliquots of the residues of evaporation are chromatographed in solvent C (table 1). To each of the two aliquots synthetically produced III and XI respectively are added. Following development the chromatogram is studied in UV light (256 nm) both before and after exposure to ammonia. The two aliquots with the reference samples are sprayed with mercuric-diphenylcarbazone reagent. From the chromatogram with the non-sprayed aliquot, regions in the same position as the reference samples are eluated. The eluates are rechromatographed and sprayed.

V: Phase E is extracted three times with 3–4 volumes of chloroform at pH 1 3. The combined chloroform phases are evaporated and two aliquots of the residue are chromatographed in solvent C. T one aliquot synthetically produced V is added. The chromatogram is studied in UV-light and sprayed.

II, VI, and VIII: Phase F is extracted as described for phase D Two aliquots are chromatographed in solvent B (table 1), one after the addition of synthetically produced II, VI, and VIII. Both aliquots are studied in *UV-light, but only the one to* which the pure samples have been added, is sprayed. From the non-sprayed aliquot the UV-absorbing regions of the reference samples are eluated. The eluates are evaporated, and the residue is dissolved in 5 ml of ether and extracted with 3.0 ml of borate buffer pH 10.8. The borate phase is used for quantitative UV-spectrophotometric analysis.

Results

Thin-layer chromatographic analyses of 24-hour urine samples from eight patients given anaesthetic doses of enibomal (500–1000 mg) intravenously did not reveal the substance in its original form. On the other hand, metabolic products were detected.

One of the metabolic products was identified as VIII by means of melting point determination and IR-spectrophotometry This product was demonstrable for up to six days after administration.

Another metabolic product was demonstrable for almost the same period. This substance could not be isolated in a sufficient amount to allow identification by means of IR-spectrophotometry TLC in three solvents (table 1) showed the substance to behave like VI. The detected substance and synthetically produced VI displayed, within the UV range, the absorption curves characteristic of N-substituted barbituric acid compounds (STUCKEY 1941). After exposure to vaporized ammonia (e g. solvent A) the absorption curves of both compounds were changed to those shown in fig. 2 This adds support to the view that the two products are identical.

The last two metabolic products were seen in the urine of some of the examined patients, but only on the first two or three days. Thin-layer chromatography showed one of these compounds to behave like VII. The other which occurred somewhat more frequently behaved chromatographically and spectrophotometrically like II None of the other theoretically possible metabolic products III V and XI were found.

Quantitative analyses of 24-hour urine samples from four of the patients showed VI and VIII to be the most important products excreted (fig. 3). The excretion of these compounds during the period of investigation constituted 6–9 and 2–7 per cent respectively of the *administered dose, whereas* the

Table 1

Thin-layer chromatography of enibomal (NFN) and metabolites.

	R_{st} values		
Solvent	A	B	C
I Enibomal	1.42	1.94	1.59
II 1 Methyl-5-acetonyl-5-isopropylbarb.	1.32	1.76	1.26
III 1-Methyl-5-carboxymethylene-5-isopropylbarb.	0.04	1.26	0.47
VI 1 Methyl-5-(2'oxo-3'-hydroxypropyl)-isopropylbarb.	0.42	1.00	0.53
V 1 Methyl-5-isopropylbarb	0.26		1.05
VII Ibomal	1.00		1.00
VIII 5-Acetonyl-5-isopropylbarb.	0.64	1.60	0.81
XI 5-Isopropylbarb.	0.05		0.30

Kieselgel GF_{254} Merck® 0.25 mm thick. Activated at 105 for 1 hour

Solvents: A n-butanol-chloroform- ammonia, aq. 25 % (120 55 15).

B diethylether-formic acid 100 % (100 0.1).

C chloroform-methanol formic acid 100 % (95 : 5 : 0.1).

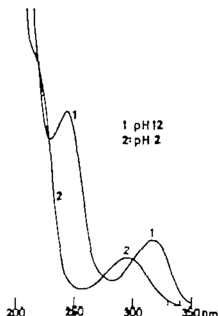


Fig. 2. Ultraviolet absorption of 1-methyl-5-(2'-oxo-3'-hydroxypropyl)-5-isopropylbarbituric acid (VI) after exposure to vaporized ammonia.

excretion of II played a minor role (0 9–0.3 per cent) VII occurred in such small amounts that quantitative determination was impossible.

Analyses of the urine samples after glucuronidase treatment, disclosed considerably higher values for VI than before this treatment. The two acetonyl compounds yielded the same or only slightly higher values after treatment with glucuronidase

Fig. 3 illustrates that the percentage of VI linked to glucuronic acid varies not only from one individual to another but also from day to day in the same individual.

Discussion

Our investigation on the metabolism of enibomal showed that the hydrolytic liberation of the bromine atom found in dogs (FREY 1959) also occurs in man. The excretion of VI demonstrated that in man enibomal also undergoes an oxidative conversion.

While VI hardly undergoes any further oxidation, owing to its high solubility in water II, the much more lipid-soluble hydrolytic product (table 2) is likely to be oxidized to another compound besides VI We have unable to confirm FREY's (1959) assumption of the presence of

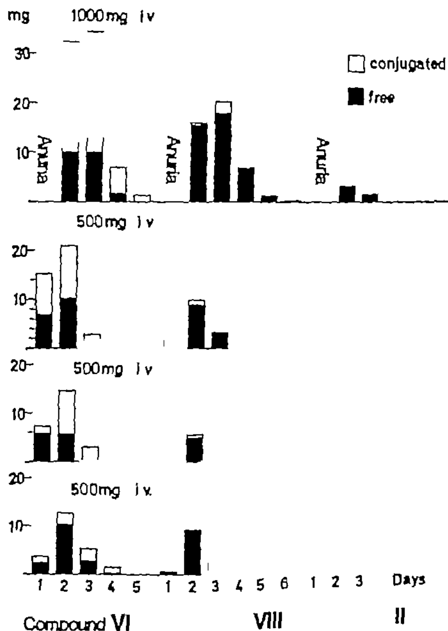


Fig. 3 The amounts of the metabolites VI VIII and II (fig. 1) excreted with the 24-hour urine by each of the 4 patients after intravenous injection of a single dose of [illegible].

oxidation product. By paper chromatography (SEIFERT 1956) the compound obtained by FREY is not identical with III or VI

Table 2

The partition coefficients diethylether/phosphate pH 7.5 (1 : 1).

I Enibomal	> 50
II 1-Methyl-5-acetonyl-5-isopropylbarbituric acid	1.67
III 1-Methyl-5-carboxymethylene-5-isopropylbarbituric acid	0.00
VI 1-Methyl-5-(2'-oxo-3'-hydroxypropyl)-5-isopropylbarb. acid	0.56
V 1-Methyl-5-isopropylbarbituric acid	0.01
VII Ibomal	> 50
VIII 5-Acetonyl-5-isopropylbarbituric acid	1.00
XI 5-Isopropylbarbituric acid	0.01

Oxidation of II may produce two reaction products. One is the above-mentioned carboxymethylene compound (III) and the other the carboxy-compound (IV). It has not yet been possible to synthetise IV (RAVN-JONSEN & HIEDS 1968) perhaps owing to steric obstruction in the molecule. If so oxidation of II into IV will result in an instantaneous decarboxylation giving V.

As we detected neither III nor V, II is unlikely to undergo oxidation in the side chain in man.

While enhexymal (NFN) is first oxidised in the cyclohexenyl ring and thereafter demethylated at the nitrogen atom (WILLIAMS & PARKE 1964), the presence of VII shows that the demethylation of enibomal can take place without previous oxidation in the substituent at carbon atom 5.

VIII may like the corresponding 1-methyl compound (II) be oxidized to a 5-carboxymethylene compound (IX) and a carboxy-compound (X) (HALBERKANN & REICHE 1927). The results of our investigation do not indicate the presence of these substances. As, however, we did not have available synthetic IX and X, we cannot definitely know whether the substances is present as a metabolic product of enibomal.

FREY (1959) compared fat and lean rats and found the sleeping time to be approximately the same for the two groups after administration of enibomal, unlike that found after administration of thiopentone (thiomebumalum NFN). FREY concluded from this, that the affinity of enibomal to fat is low. However, since enibomal is readily soluble in ether at about the physiological pH (table 2), the compound must also be assumed to be easily soluble in fat.

The observation made by FREY may thus be due either to enibomal taking a longer time than thiopentone to be absorbed in fatty tissue or to the awakening being due to a rapidly proceeding metabolism of the drug.

Both FREY's and our own investigations showed that deposition takes place. The partition coefficients seen in table 2 show that it must be the parent substance which is deposited. No more than 8–16 per cent of the

stered dose however was recoverable. We therefore cannot give an opinion on the influence of deposition on the short-term action of enibomal. That a maximum excretion of VI and VIII was not attained until the second or third day after the administration, indicates that these compounds cannot be the final products in a metabolic process which determines the short-term action of enibomal.

Acknowledgement

The present study was aided by grants from Reinholdt W Jorck og hustrus Fond and Statens Almindelige Videnskabsfond. The author wishes to thank Professor Ole Secher M D., for the great interest he has displayed in the work. The author is also grateful to Dr Kirsten Klose Mikkelsen and Dr Brynjolf Petersen for their help in collecting the test material Mrs. Jane Tullberg is thanked for her valuable technical assistance.

REFERENCES

Frey H. H., Vergleichende Untersuchungen zum Stoffwechsel intravenöser Kurznarkotika. *Arch. Int Pharmacodyn.* 1959 118 12–61.

Halberkann, J & F Reiche: Über die Ausscheidung einiger viel verwendeter Barbitursäureverbindungen mit dem Urin. *Münch. Med. Wochensch.* 1927 74, 1430–32.

Ravn-Jonsen, A. & H. Hjeds: Synthesis and molecular structure of some new barbituric acids. *Dansk Tidsskr Farm.* 1968, 42, 289–95

Seifert, R., Papierchromatographische Erfassung einiger wichtiger Barbitursäure-Derivate. *Arzneimittel-Forsch* 1956, 16, 413–14.

Smith, L. Chromatographic and electrophoretic technique. William Heinemann Ltd. *Sec. Edt.* 1960, vol. I, p. 386.

Stuckey R. R., The ultra-violet absorption spectra of barbituric acid derivatives, part II, barbitone and phenobarbitone and their methyl-derivatives. *Quart. J Pharm. Pharmacol.* 1941 14 213–25.

William, R. T & P V Parke: The metabolic fate of drugs. *Ann. Review of Pharmacol.* 1964 4 85–114.

Acta pharmacol et toxicol 1970 28 493–498

From the Department of Pharmacology University of Uppsala, Uppsala, Sweden[1] and the Department of Pharmacology University of Saarland, Homburg, Germany[2]

Unequal Anaesthetic Potency Despite Equal Brain Concentration of Hexobarbital Antipodes

By

Göran Wahlström[1] Horst Büch[2] and Walter Buzello[2]

(Received March 2, 1970)

Abstract Confirming earlier results, approximately 100 mg/kg more (−)- than (+)-hexobarbital (infused intravenously) was needed to obtain "silent second" (= burst suppression of 1 sec. or more in the EEG) in male rats. After infusion of (+)- (−)- and racemic hexobarbital at exactly the same infusion rates and in doses which with the racemate gave a silent second, no difference was found in the brain concentrations. The amount of hexobarbital infused was, however in the case of the (+)-antipode approximately 20 mg/kg higher and in the case of the (−)-antipode, approximately 80 mg/kg lower than the dose which gave silent second with the corresponding antipodes. It is thus highly unlikely that different rates of accumulation in the central nervous system during the infusion would explain the differences in potency between the two antipodes. The 28 % lower concentration of (+)-hexobarbital compared to (−)-hexobarbital found in the liver could be explained by earlier findings i. e. that the (+)-antipode is more rapidly metabolized than the (−)-antipode.

Key-words. Hexobarbital – rats – brain – electroencephalography – isomers.

The optical antipodes of hexobarbital (enhexymalum NFN methylcyclohexenyl N-methyl-barbituric acid) have different anaesthetic properties (Wahlström 1966b Rummel *et al.* 1967). When infused slowly considerably less of (+)-hexobarbital as compared to (−)-hexobarbital is needed to obtain a burst suppression of 1 sec. or more in the EEG (Wahlström 1966b). Rummel *et al.* (1967) have studied the distribution patterns of hexobarbital in rats and found a larger brain/serum ratio i. e. concentration in brain/concentration in serum for (+)-hexobarbital than for (−)-hexobarbital after rapid intravenous injection. Even if the difference was mainly due to different concentrations of (+)- and (−)-hexobarbital in the serum, this could mean a more selective accumulation of (+)-hexobarbital in the CNS and might possibly explain the difference found in anaesthetic threshold. If the observed difference was due to a selective accumulation of one hexobarbital antipode

difference in the brain concentration would be found providing similar amounts of the antipodes are infused with the infusion technique used in the threshold determinations. This has been done in the present experiments.

Methods

Experimental.

Male Sprague-Dawley rats weighing around 350 g were used in the present experiments. They were kept on an inverted light darkness schedule (darkness 8 to 20) and were well adapted to this rhythm before the experiments. With these nocturnal animals all experiments were thus performed during the period of activity.

Threshold determinations were performed as described in detail elsewhere (WAHLSTRÖM 1966a). Hexobarbital as the optical antipodes or the racemate was infused into a tail vein at a constant rate of 0.25 mg/kg/sec. (the volume rate was 0.1 ml/min.) and the EEG was recorded during the infusion. The dose needed to obtain a burst suppression of 1 sec. or more (the silent second) was used as the threshold dose.

In experiment 1 two threshold determinations with the racemate were performed (with rats previously not used in any experiments) before the experiments with the optical antipodes were performed. The first threshold determination was discarded and the second one was used to ensure that all the animals used had a threshold with racemic hexobarbital within the range obtained in previous experiments (WAHLSTRÖM 1966a). The rats were then divided into two groups and the dose needed to obtain the silent second with the optical antipodes was determined.

In experiment 2 three threshold determinations were performed with the racemate in rats previously not used in any experiments. The first one was discarded and the other two used to calculate an average racemate threshold dose for the individual rat. The interval between the threshold determinations in the same animal was at least two days. The dose for silent second is not systematically influenced by threshold determinations performed within this interval (WAHLSTRÖM 1966b). The thresholds are furthermore not influenced by a pentobarbital (mebumalum NFN) induced increase in the metabolism of hexobarbital (WAHLSTRÖM 1968).

The animals were then randomly divided into three groups. Two groups were infused with (+)- or (−)-hexobarbital up to the average racemate threshold and the third group was infused with the racemate up to the silent second. The abdomen was then immediately opened and a venous blood sample was obtained in a heparinized tube. The average times between the start of the infusion and the time at which the animals were killed were found to be as follows: group infused with (+)-hexobarbital 301 sec., group infused with (−)-hexobarbital, 288 sec., and group infused with the racemate 300 sec. The brain and part of the liver were then removed and frozen on dry ice within 4–6 min. after the end of the infusion. The brain, liver and serum samples were then kept frozen until analysed. With this experimental design a selective accumulation will become evident as a difference in brain concentration which can be statistically evaluated.

(+)- and (−)-hexobarbital (as the acid) were prepared according to KNABE & KRÄUTER (1965). The antipodes used in the experiments had $(\alpha)^{20}_{D}$ + 11.8 and − 12.3 and the melting points (uncorrected) were 153.5 and 152.3 respectively. Except for optical activity they are physico-chemically identical. Immediately before use they are dissolved in 0.5 N NaOH (0.186 mg NaOH/mg of hexobarbital as the acid). Racemic hexobarbital was obtained as the sodium salt from Bayer AB. All further dilutions

were performed as previously described (WAHLSTRÖM 1966b). All the doses are given as the sodium salt.

Isolation and determination f hexobarbital.

A defined volume of serum in the range of 1–2 ml was acidified to pH 4.6 by M/15 KH_2PO_4 solution and twice extracted by 10 ml of diethylether The ether phases were pooled and evaporized in a water bath at 37° The residue was dissolved in acetone/ether (1/1) and spotted quantitatively on a thin layer chromatography (TLC) plate.

The whole of the *brain* and about 2 g of liver were taken for analysis. The specimens were weighed and homogenized in 8 ml of 0.2 M tris buffer pH 8.2. The homogenates were centrifuged (20000 × g for 15 min.) and the supernatant fractions separated. The residues were suspended in another 8 ml portion of buffer and then centrifuged again. The supernatant fractions of both turns were pooled. After acidification with 0.2 ml of 5 N-HCL the solutions were extracted twice with 50 ml of chloroform. Both chloroform phases were pooled and evaporated (40 rotation evaporizer BÜCHI) The residue were dissolved in methanol and spotted quantitatively upon TCL plates. In all cases a hexobarbital solution in methanol was chromatographed simultaneously as reference.

Chromatographic processing and determination.

TCL plates coated with silica gel GF 254 (MERCK) according to STAHL's method (1962) were used. Processing was performed in isopropranol/chloroform/ammonia 33 % (60/40/10) as mobile phase. Under UV-light (Mineralight) hexobarbital was detected as either fluorescence extinction spots or zones. After identification by means of refer ence spots the silica gel of the hexobarbital spots was removed from the plates in centrifugation tubes and then eluted with 4 0 ml of 0.05 N-NaOH. After short period of centrifugation the silica gel was sedimented. The hexobarbital concentration was determined photometrically at 245 mμ (spectral photometer PQM II Zeiss).

Results

Fig. 1 shows the results of two experiments. It is seen that in experiment 1 there was a large difference in the amounts of (+)- and (−)-hexobarbital needed to obtain the silent second. In experiment 2 the doses infused were similar in all groups. A silent second had, however occurred in all the animals which had been infused with (+)-hexobarbital when the infusion was stopped The amount needed to obtain this silent second is shown by the hatched portion of the (+)-bar and was of the same magnitude as in ex periment 1. No silent second was obtained in the animals which received (−)-hexobarbital.

The average hexobarbital concentration in the serum was 22.1 μg/ml (28.2 /) lower in the (+)-hexobarbital treated animals than in the (−)-hexobarbital treated animals. The corresponding figure for the concen trations in the liver was 26.2 μg/g (27 6 /) A statistical analysis with Student's t-test gave a significant difference between the concentration of (+)-hexobarbital and (−)-hexobarbital in the serum (P less than 0 0

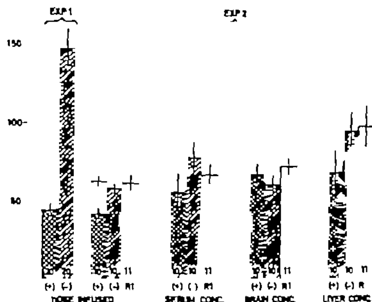


Fig. 1 Averages of amounts infused and concentrations (CONC.) of hexobarbital in the serum, brain and liver. In experiment 1 (EXP 1) the animals were infused up to the silent second. In experiment 2 (EXP 2) (+)- and (−)-hexobarbital were infused up to a pre-determined amount corresponding to the amount needed to obtain the silent second with the racemate in an earlier test. In group R1 the racemate was infused up to the silent second. The lower line in the bar of dose infused with (+)-hexobarbital indicates the amount needed to obtain the silent second, the upper line denotes the amount infused. Twice the standard error is indicated at the top of the bars and the number of animals at the bottom of the bars. The scale on the ordinate is µg/g (dose infused), µg/ml (concentration of hexobarbital in the serum) and µg/g (concentration of hexobarbital in the brain and liver).

significant difference between the concentration of (+)-hexobarbital and (−)-hexobarbital in the liver tissue was also demonstrated (P less than 0.02). By chance the amount of (+)-hexobarbital happened to be 4.8 mg/kg (8.3 %) larger than the amount of (−)-hexobarbital infused. A similar difference in the brain concentrations (6.5 µg/g, 10.7 %) could be explained by this occurrence. None of the last two differences reached a P value smaller than 0.05 (Student's t test).

Discussion

From the data presented in fig. 1 an important conclusion regarding the brain concentrations of the optical antipodes at the silent second can be obtained. In experiment 2 approximately 20 mg/kg more (+)-hexobarbital than that needed to obtain the silent second was infused. It follows from

experiment 1 that approximately 80 mg/kg more (−)-hexobarbital would have been needed to obtain a silent second in experiment 2. Thus, the dose of (−)-hexobarbital was far below that needed for silent second. Nonetheless there was only a small and not significant difference in the brain concentrations found in the (+)- and (−)-hexobarbital groups of experiment 2, in which the doses of (−)- and (+)-hexobarbital infused were similar. It has been convincently demonstrated that the brain concentration needed for the silent second must be lower for (+)-hexobarbital than for (−)-hexobarbital. A comparison between the (+)-hexobarbital group and group R1 (infused with the racemate to obtain the silent second) further strengthens this point. The (+)-hexobarbital group was infused with approximately 20 mg/kg (= 30 %) more hexobarbital than needed in order to obtain the silent second. Nonetheless the average brain concentration in the (+)-hexobarbital group was if anything lower than the brain concentration in group R1 where the animals were killed at the silent second. The amounts of hexobarbital infused were similar in these two groups.

The difference in anaesthetic potency of the antipodes of hexobarbital with regard to the silent second could thus not be explained by a stereospecific penetration process which was a possibility indicated by the earlier differences found in brain/serum ratios (RUMMEL *et al.* 1967). A stereospecific process inside the brain must be involved to explain at least in part the differences between the activities of the optical antipodes of hexobarbital. This also seems to be the case with regard to methylphenobarbital (enphenemalum NFN) (BÜCH *et al.* 1968) where the less active (+)-methylphenobarbital reached higher brain concentration, without causing a loss of the righting reflex, than the more active (−)-methylphenobarbital where the righting reflex was lost.

RUMMEL *et al.* (1967) have shown in non-pretreated rats and in rats pretreated with phenobarbital (induced) that (+)-hexobarbital is more rapidly metabolized than (−)-hexobarbital. This has been confirmed in studies on microsome preparations from rat liver which showed that the (+)-hexobarbital was more rapidly degradated than the (−)-hexobarbital (DEGKWITZ *et al.* 1969, FURNER *et al.* 1969). This difference cannot explain the difference in amounts of the optical antipodes needed to obtain the silent second – it would be expected to work in the opposite direction. However it could explain the difference in the concentration of unmetabolized drug in the liver seen in fig. 1.

Acknowledgement

The optical isomers of hexobarbital used in this study were separated by docent L. Terenius in the Department of Pharmacology at Uppsala and

kind help is gratefully acknowledged. Thanks are also due to Mr T Ekwall for skilful technical assistance.

Racemic hexobarbital sodium (evipan®) was generously supplied by Bayer AG through Mr R. Arensberg.

This study was supported by the Tri-centennial fund of the Bank of Sweden.

REFERENCES

Büch, H., W Buzello, O Neurohr & W Rummel. Vergleich von Verteilung, narkotischer Wirksamkeit und metabolischer Elimination der optischen Antipoden von *Methylphenobarbital. Biochem. Pharmacol.* 1968, 17 2391–2398.

Degkwitz, E., V Ullrich, H. Staudinger & W Rummel Metabolism and cytochrome P-450 binding spectra of (+)- and (−)-hexobarbital in rat liver microsomes. *Hoppe-Seyler's Z. Physiol. Chem* 1969 350 547–553.

Furner R. L., J S. McCarthy R. E. Stitzel & M. W Anders: Stereoselective metabolism of the enantiomers of hexobarbital. *J Pharmacol. Exp Therap* 1969 169 153–158.

Knabe, J & R. Krämer Spaltung von Hexobarbital (Evipan®) in optische Antipoden. *Arch. Pharm.* 1965 298, 1–4.

Rummel, W U Brandenburger & H. Büch. Versuche zur Aufklärung der Ursachen der unterschiedlichen narkotischen Wirksamkeit von (+)- und (−)-Hexobarbital. *Med. Pharmacol. exp* 1967 16 496–504

Stahl, E. *Dünnschichtchromatographie.* Springer Verlag, Berlin-Göttingen-Heidelberg 1962, 7–10.

Wahlström, G Estimation of brain sensitivity to hexobarbitone (enhexymal NFN) in rats by an EEG threshold. *Acta pharmacol et toxicol.* 1966a, 24 404–418.

Wahlström, G Differences in anesthetic properties between the optical antipodes of hexobarbital in the rat. *Life Sciences* 1966b, 5 1781 1790

Wahlström, G Hexobarbital (enhexymalum NFN) sleeping times and EEG threshold doses as measurements of tolerance to barbiturates in the rat. *Acta pharmacol. et toxicol.* 1968, 26, 64–80.

Acta pharmacol et toxicol. 1970 28, 499–506.

From the Industrial Toxicology Research Centre, Lucknow U P India

Effect of Benzanthrone on the Body Level of Ascorbic Acid in Guinea Pigs

By

K. P Pandya, G. B. Singh and N. C. Joshi

(Received April 30, 1970)

Abstract: The effect of benzanthrone on the blood, adrenal and liver ascorbic acid levels in adult male guinea-pigs has been investigated. Benzanthrone and ascorbic acid were administered intraperitoneally and orally in doses of 25 mg and 50 mg/kg body weight respectively Benzanthrone alone caused significant decrease of ascorbic acid levels in the blood, adrenal and liver Supplementation of ascorbic acid appreciably restored the blood ascorbic acid. However adrenal and liver ascorbic acid levels were restored only to some extent. Histochemical examination of ascorbic acid in the adrenal revealed almost similar changes. The mortality rate due to benzanthrone toxicity (500 mg/kg) was lowered by 40 % in non-scorbutic as compared to scorbutic guinea-pigs.

Key-words: Benzanthrone – ascorbic acid.

It has been observed in a dye producing factory that workers who come in contact with the dye benzanthrone (a derivative of anthraquinone) during its manufacture, pulverization and storage develop itching, burning sensation erythema and pigmentation on exposed and even covered parts of the skin. In a few cases, pigmentation spreads rapidly SINGH *et al.* (1967) studied the effect of local application of benzanthrone on the skin of mice and observed dermal changes when the animals were exposed to sunlight. In their preliminary *report on benzanthrone toxicity* SINGH & ZAIDI *(1969) observed that* oral administration of ascorbic acid partially inhibited the benzanthrone-induced dermal changes in experimental animals. Since ascorbic acid appears to protect the animals from the dermal toxicity of the dye, it was of interest to study the toxic action of the dye in scorbutic animals and the reversal of the toxic symptoms by ascorbic acid.

Material and Methods

Animals

One hundred male guinea-pigs of I. T R. C. colony average weight 400 grams, used.

Plan of experiment.

1. *Body level ascorbic acid studies.* The animals which were kept on routine stock diet consisting of gram, bran and green leafy vegetables, were divided into four groups of ten each, as follows.

Group I. Normal control.

Group II. In this group 25 mg/kg body weight of benzanthrone, suspended in 1 ml normal saline, was injected intraperitoneally once a day

Group III. In this group 50 mg/kg body weight of ascorbic acid, dissolved in 1 ml distilled water was fed orally daily through a No. 5 rubber catheter

Group IV The animals were treated as in group III and 25 mg/kg body weight of benzanthrone in suspension was administered as in group II.

Animals of all the groups were sacrificed after 7 days by ether anaesthesia. Approximately 5 ml blood were drawn directly from the heart. The abdomen of each animal was opened and the liver and adrenals were dissected out. Histochemical examination of ascorbic acid in one of the adrenals (left) was done by the method of BACCHUS (1950). Estimation of the total ascorbic acid content of the blood, liver and the right adrenal was done by the method of SCHAFFERT & KINGSLEY (1955).

2. *Study of mortality rate.* Sixty male guinea pigs were divided into three groups of 20 each, I) normal II) scorbutic and III) non-scorbutic. Routine stock diet was fed to the animals of group I. Animals of groups II and III were fed a synthetic scorbutic diet (KAW *et al.* 1969) consisting of crushed barley 64 parts, crushed gram (CICER ARIETINUM), 20 parts, casein, 12 parts, calcium carbonate, 3 parts, and sodium chloride, 1 part, vitamins A and D (2,400 and 400 I.u. respectively) were supplied twice weekly 25 mg/kg body weight/day of ascorbic acid was fed to group III. After 15 days, 500 mg/kg body weight of benzanthrone in normal saline was injected intraperitoneally into each animal of all the groups. The mortality rate was recorded within twentyfour hours. The dose of benzanthrone was selected on the basis of our preliminary investigation which showed that 500 mg/kg body weight was sufficient to kill 50 per cent of stock diet fed animals in 24 hours.

Results

Histochemical studies.

Histochemical examination of ascorbic acid was done only in the adrenal because its concentration in the liver of various groups of animals was not sufficient to be visualised. The adrenal of animals belonging to groups I and III showed cells densely filled with compact ascorbic acid granules (figs. 1 & 2). In groups II and IV however the number of ascorbic acid granules was scanty They were discrete smaller in size and irregularly scattered in the cytoplasm (figs. 3 & 4)

Estimation of ascorbic acid

The results are given in table 1

Ascorbic acid levels in the blood, adrenal and liver were significantly lowered ($P < 0.01$) in the benzanthrone treated animals of group II as com-



Fig. 1. Normal control. Densely compact ascorbic acid granules. Silver stain. Magnification × 120.

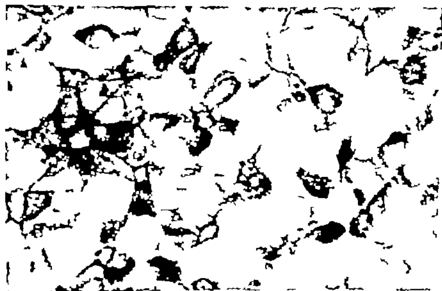


Fig. 2. Ascorbic acid administered. Appearance similar to that in fig. 1. Silver Magnification × 120.

Fig. 3. Benzanthrone only. Ascorbic acid granules are discrete, smaller in size and irregularly scattered. Silver stain. Magnification × 120.

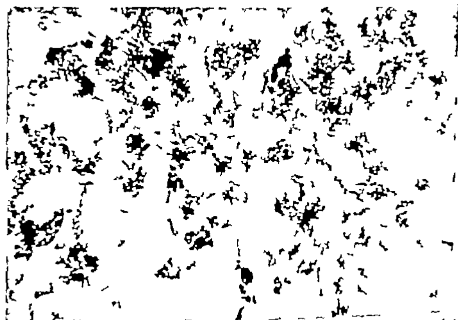


Fig. 4. Benzanthrone and ascorbic acid administered. Almost similar appearance to that observed in fig. 3. Silver stain. Magnification × 120.

Table 1

Ascorbic acid content of the blood, adrenal and liver in control and experimental animals. Comparisons between groups have been made from a pooled estimate of the variance, for each tissue (after analysis of variance).

	Blood (mg/100 ml) Mean ± S. E. M	Adrenal (mg/100 gram of wet wt.) Mean ± S. E. M.	Liver (mg/100 gram of wet wt.) Mean ± S. E. M.
Group I (Normal control)	1.161 ± 0.083	95.7 ± 6.4	21.81 ± 0.89
Group II (Benzanthrone administered)	0.430 ± 0.013	48.9 ± 4.2	17.88 ± 0.85
Group III (Ascorbic acid administered)	1.180 ± 0.090	100.4 ± 9.3	20.95 ± 0.94
Group IV (Benzanthrone & ascorbic acid administered)	0.982 ± 0.062	62.4 ± 2.3	19.53 ± 1.11

pared to the control group I. However significant differences were not observed in the ascorbic acid contents of these tissues after administration of ascorbic acid in group III as compared to the controls. Ascorbic acid supplementation in benzanthrone treated animals (group IV) appreciably restored the blood ascorbic acid level ($P < 0.01$) as compared to animals of group II. However in the case of the adrenal and liver, the ascorbic acid level was restored to some extent only

Mortality rate.

The results are given in table 2. In animals supplemented with ascorbic acid (25 mg/kg/day) the mortality rate was lowered to the extent of 40 % as compared to animals of the scorbutic group. Scorbutic animals appeared to be completely unprotected due to the lack of ascorbic acid.

Discussion

MURAKAMI (1939) demonstrated that a diet deficient in ascorbic acid lowered the detoxication mechanism of guinea-pig liver but this condition was reversed by supplementation with ascorbic acid. BUNDESEN et al showed that the reaction of hypersensitive patients can be pre

Table 2

Mortality rate in normal and synthetic diet fed scorbutic and non-scorbutic guinea-pigs. Mortality rate was lowered by 40 per cent in non-scorbutic as compared to scorbutic guinea-pigs.

	No. of animals	No. of animals died within 24 hours of benzanthrone administration (500 mg/kg)	Mortality rate
Group I Normal (Stock diet)	20	10	50 per cent
Group II Scorbutic (synthetic scorbutic diet)	20	20	100 per cent
Group III Non-scorbutic (Synthetic scorbutic diet with additional ascorbic acid)	20	12	60 per cent

ascorbic acid content of the blood is maintained at a sufficiently high level. BEYER (1943) found that the hepatic toxic effect of hydrazine was reduced by ascorbic acid and enhanced by ascorbic acid deficiency The mechanism of action was not explained but it was suggested that ascorbic acid exerts its effect indirectly by allowing normal metabolism of carbohydrate and protein.

Ascorbic acid has also been reported to be effective in allergy sensitivity and toxicity of the skin due to various synthetic dyes and its usefulness in the prevention of dermal lesions caused by the local application of benzanthrone was shown by SINGH & ZAIDI (1969). Ascorbic acid has been found useful in the prevention of chrome dermatitis (SAMITZ *et al* 1968). WAPNICK *et al* (1968) observed a decrease in white blood cell ascorbic acid levels in subjects suffering from iron toxicity

FORSSMAN & FRYKHOLM (1947) observed that exposure to benzene produced an increased requirement of ascorbic acid. EDDY (1949) reported that ascorbic acid might exert a protective action against benzene poisoning. Recently the effect of chronic benzene poisoning on ascorbic acid levels in the guinea-pig and rat was studied by IANCU *et al* (1969). In the guinea-pig, benzene poisoning lowered ascorbic acid levels in the blood and organs (adrenals, liver) and intensified ascorburia. The toxic effect was *attenuated* by the administration of ascorbic acid at a dose of 6 mg/day and the mortality rate was lowered by 57 / They concluded that benzene affected the metabolism of ascorbic acid by causing an increase in catabolism.

In the present experiments, administration of benzanthrone caused a significant decrease of ascorbic acid level in the blood, adrenal and liver Supplementation with ascorbic acid had only a slight effect on the levels in the adrenal and liver but restored the blood ascorbic acid. This would suggest an increased requirement of ascorbic acid in guinea-pigs after the administration of benzanthrone. Similar conclusions were also obtained from studies of mortality rates. A 40 / lowering of the mortality rate after supplementation of ascorbic acid (25 mg/kg/day) as compared to animals of the scorbutic group also emphasizes the protective role of the vitamin in cases of benzanthrone toxicity These studies suggest the possible usefulness of ascorbic acid in clinical therapy particularly of workers who are exposed to benzanthrone and develop various dermal symptoms.

Acknowledgements

The authors are grateful to Dr S. H. Zaidi, Director Industrial Toxicology Research Centre for his keen interest in the work. Technical assistance of Messrs. Mulkraj Kanahiya Lal and V G Misra is highly appreciated. Thanks are due to Mr Musleh Ahmad for the preparation of photomicrographs and Mr P A. George for the statistical analysis.

REFERENCES

Becchus, H. Cytochemical study of the adrenal cortex of the rat under salt stresses. *Amer J Physiol.* 1950, 163, 326–331.

Beyer K. H. Protective action of vitamin C against experimental hepatic damage. *Arch Intern. Med.* 1943, 71, 315–324.

Bundesen, H. H., H. C. S. Aron, R. S. Greenbaum, C. J Farmer & A. F Abt: The detoxifying action of vitamin C (ascorbic acid) in arsenical therapy I. Ascorbic acid as preventive of reactions of human skin to neoarsphenamine. *J Amer Med. Assoc* 1941 117 1692–1695.

Eddy W H *Vitaminology the chemistry and function of the vitamins.* The Williams and Wilkins Company Baltimore, 1949 pp 306–307

Forssman, S. & K. O. Frykholm. Benzene poisoning II. Examination of workers exposed to benzene with reference to the presence of ethereal sulfate, muconic acid, urochrome A and polyphenols in the urine together with vitamin C deficiency Prophylactic measures. *Acta med. Scandinav* 1947 128, 256–280.

Iancu, G S. Dumitrache, A. Rujinski & M. Draghiciescu Effect of chronic benzene poisoning on vitamin C levels in the guineapig and rat. *Igiena* 1969 18, 1 11.

Kaw J L. & S. H. Zaidi: Effect of ascorbic acid on pulmonary silicosis of guineapigs. *Arch. Environ. Hlth.* 1969 19 72–82.

Murakami, O The influence of ascorbic acid upon the liver function and the mutual relation between vitamins B_1 and C. I Influence of ascorbic acid upon ment-excreting function of the liver *Jap J gastroenterol.* 1939 11 1–6

Table 2

Mortality rate in normal and synthetic diet fed scorbutic and non-scorbutic guinea-pigs. Mortality rate was lowered by 40 per cent in non-scorbutic as compared to scorbutic guinea-pigs.

	No of animals	No. of animals died within 24 hours of benzanthrone administration (500 mg/kg)	Mortality rate
Group I Normal (Stock diet)	20	10	50 per cent
Group II Scorbutic (synthetic scorbutic diet)	20	20	100 per cent
Group III Non-scorbutic (Synthetic scorbutic diet with additional ascorbic acid)	20	12	60 per cent

ascorbic acid content of the blood is maintained at a sufficiently high level. BEYER (1943) found that the hepatic toxic effect of hydrazine was reduced by ascorbic acid and enhanced by ascorbic acid deficiency The mechanism of action was not explained but it was suggested that ascorbic acid exerts its effect indirectly by allowing normal metabolism of carbohydrate and protein.

Ascorbic acid has also been reported to be effective in allergy sensitivity and toxicity of the skin due to various synthetic dyes and its usefulness in the prevention of dermal lesions caused by the local application of benzanthrone was shown by SINGH & ZAIDI (1969) Ascorbic acid has been found useful in the prevention of chrome dermatitis (SAMITZ *et al* 1968). WAPNICK *et al* (1968) observed a decrease in white blood cell ascorbic acid levels in subjects suffering from iron toxicity

FORSSMAN & FRYKHOLM (1947) observed that exposure to benzene produced an increased requirement of ascorbic acid. EDDY (1949) reported that ascorbic acid might exert a protective action against benzene poisoning. Recently the effect of chronic benzene poisoning on ascorbic acid levels in the guinea-pig and rat was studied by IANCU *et al* (1969). In the guinea-pig benzene poisoning lowered ascorbic acid levels in the blood and organs (adrenals, liver) and intensified ascorburia. The toxic effect was attenuated by the administration of ascorbic acid at a dose of 6 *mg/day* and the mortality rate was lowered by 57 / They concluded that benzene affected the metabolism of ascorbic acid by causing an increase in catabolism.

Samitz, M. M., D. M. Scheiner & S. A. Katz: Ascorbic acid in the production of dermatitis, mechanism of inactivation of chromium. *Arch. Environ. Hlth.* 1. 17 44–45.

Schaffert, R. R. & G. R. Kingsley: A rapid, simple method for the determination of reduced, dehydro and total ascorbic acid in biological material. *J. Biol. Chem.* 1955 212, 59–68.

Singh, G. B., S. N. Sharma & S. H. Zaidi. Effect of benzanthrone on the skin of mice. *Ind. J. Med. Sci.* 1967 21, 727–728.

Singh, G. B. & S. H. Zaidi. Preliminary clinical and experimental studies on benzanthrone toxicity. *J. Ind. Med. Assoc.* 1969 52, 558–560.

Wapnick, A. A., S. R. Lynch, P. Krawitz, H. C. Seftel, R. W. Charlton & T. H. Bothwell. Effect of iron overload on ascorbic acid metabolism. *Brit. Med. J.* 1968, 3, 704